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(54) Title: ADENOVIRAL VECTORS FOR TREATING DISEASE

(57) Abstract: Adenoviral vectors, including mutant adenoviruses, that have restriction sites in the E3 region, that facilitate its partial or total deletion, or select genes contained therein, and compositions and methods for substituting heterologous gene(s), if desired, which gene(s) will exhibit an expression pattern, both in terms of timing and degree of expression, similar to the endogenous adenoviral gene that it replaces, and further optionally including mutations in other parts of the adenoviral genome, including certain E1B or E1A regions, and that have applications for diagnosing or treating disease, preferably disease involving unwanted cell growth, including cancer.

ADENOVIRAL VECTORS FOR TREATING DISEASEField of the Invention

The invention described herein relates generally to the field of gene therapy, and more specifically to adenoviral vectors that have prophylactic or therapeutic applications.

Background of the Invention

Adenovirus is a vector of choice for performing gene therapy. See, Jolly, D.. Cancer Gene Therapy, vol. 1, no. 1, 1994: pp51-64. The well-characterized molecular genetics of adenovirus render it an advantageous vector in this regard. Adenoviruses are nonenveloped icosohedral double-stranded DNA viruses with a linear genome of approximately 36 kilobase pairs. Each end of the viral genome has a short sequence known as the inverted terminal repeat (or ITR), which is required for viral replication. Portions of the viral genome can be readily substituted with DNA of foreign origin, and furthermore, recombinant adenoviruses are structurally stable.

The adenovirus replication cycle has two phases: an early phase, during which 4 transcription units E1, E2, E3, and E4 are expressed, and a late phase which occurs after the onset of viral DNA synthesis when late transcripts are expressed primarily from the major late promoter (MLP). The late messages encode most of the virus's structural proteins. The gene products of E1, E2 and E4 are responsible for transcriptional activation, cell transformation, viral DNA replication, as well as other viral functions, and are necessary for viral growth.

To date most adenoviral vectors are based on viruses mutated in E1, E3 or a site upstream of E4 which provide for sites for the insertion of foreign DNA. The majority of vectors are based on adenovirus mutants which lack the E1 region of the genome. By deleting this region, the virus is rendered replication incompetent while simultaneously allowing for the insertion of foreign genes.

There are numerous reports on the use of adenovirus for gene therapy. For example, Smith, et al., Nature Genetics, Vol. 5, pgs. 397-402 (1993) discloses the administration to mice of an adenoviral vector including a human Factor IX gene. Such administration resulted in efficient liver transduction and plasma levels of human Factor IX that would be therapeutic for hemophilia B patients. Human Factor IX levels, however, slowly declined to baseline by nine weeks after injection, and were not re-established by a second vector injection. Smith, et al., also found that neutralizing antibodies to adenovirus block successful repeat administration of the adenovirus.

Kozarsky, et al., J. Biol. Chem., Vol. 269, No. 18, pgs. 13695-13702 (May 6, 1994) discloses the infusion of an adenoviral vector including DNA encoding the LDL receptor to rabbits. Stable expression of the LDL receptor gene was found in the rabbits for 7 to 10 days, and diminished to undetectable levels within 3 weeks. The development of neutralizing antibodies to the adenovirus resulted in a second dose being completely ineffective.

Kass-Eisler, et al.. Gene Therapy, Vol. 1, pgs. 395-402 (1994) suggest that a T-cell response contributes to, but is not solely responsible for, the limited duration of expression in adults from adenovirus vectors. The authors further show that cyclosporin A is not effective in blocking the humoral response to the vector.

5 Fang, et al.. J. Cell. Biochem.. Supplement 21A, C6-109, pg 363 (1995) disclose the attempted re-injection of an adenovirus vector in dogs that were treated with cyclosporin A, an immunosuppressive agent. Such attempted re-injection was unsuccessful.

Yang, et al., Proc. Nat. Acad. Sci., Vol. 91, pgs. 4407-4411 (May 1994) describe recombinant adenoviruses in which the E1a and E1b regions have been deleted. Such viruses also include a 10 transgene. When these adenoviruses are administered to an animal host, cells harboring the recombinant viral genome express the transgene as desired; however, low level expression of viral genes also occurs.

As exemplified above, adenoviruses can be efficient in gene transfer into cells in vivo, and thus may be employed as delivery vehicles for introducing desired genes into eukaryotic cells. There are, however, several limitations to adenovirus gene transfer which are due in part to host responses directed 15 at either the adenovirus vector particle, breakdown products of the vector particle, or the transduced cells. These host responses include non-specific responses and specific immune responses. The non-specific responses include inflammatory and non-inflammatory changes. An example of the latter is a change in host cell gene expression. Specific immune responses include various cellular responses and humoral antibody responses. Cellular responses include those mediated by T-helper lymphocytes, T-suppressor lymphocytes, cytotoxic T lymphocytes (CTL), and natural killer cells.

Despite the high efficiency of adenovirus vector mediated gene transfer, the transient nature of adenovirus vector mediated gene transfer has suggested that repeat administrations of adenovirus vectors 25 may be necessary. Recent studies in cotton rats, however, have demonstrated that host immune responses directed towards adenoviral vectors correlate with decreased efficiency of gene transfer and expression after repeated administration. Yei et al., Gene Therapy, 1:192-200 (1994). The E3 region encodes several immunoregulatory proteins, which are not required for viral replication: gp19K, 10.4K, 14.5K and 14.7, and one protein, 11.6K, that is required for lysis of infected cells, and release of infectious progeny. Additionally, the E3 region also contains open reading frames for two proteins, 12.5k and 6.7k, whose functions have yet to be identified.

30 While the E3 region is not essential for viral replication, it does play a key role in modulating the host immune or inflammatory responses to the virus. For instance, in the case of the immune response it is known that gp19K binds to MHC class I molecules in the endoplasmic reticulum, thus inhibiting its glycosylation and transport to the surface of the virally infected cells. Consequently, the infected cells are not recognized as foreign by cytotoxic lymphocytes. See, Burgert, B., et al., Proc. 35 Natl. Acad. Sci USA 1987; vol. 8: 1356-60.

Because of the many functions of the E3 region, it would be desirable to have an adenoviral vector for gene therapy applications that would permit one to delete particular regions of E3, and substitute foreign DNA, depending on the intended application of the vector. For example, there are described deletions in the E3 region that result in the removal of 1.88 kb between the XbaI sites. See,  
5 Berkner, K. and Sharp, P., (1983) Nucleic Acids Res. Vol. 11, pages 6003-6020, and Haj-Ahmad, Y. and Graham, F. (1986) J. Virol. Vol. 57, pages 267-274. Further, there is described compositions and methods for constructing adenovirus having insertions or deletions in both the E1 and E3 regions. See also, Ginsberg, H.S. et al., Proc. Natl. Acad. Sci. USA 1989, vol. 86, pp. 3823-7.

Thus, while these vectors have mutations in the E3 region, or large parts of the region deleted, to date there does not exist a vector that allows one to remove select parts of the E3 region and substitute foreign DNA.  
10

#### Summary of the Invention

A first object of the invention is to describe recombinant adenoviral vectors that have restriction sites in the E3 region that facilitate partial or total deletion of this region, or select genes contained  
15 therein, and if desired, substitute a heterologous gene, which gene will exhibit an expression pattern, both in terms of timing and degree of expression, substantially similar to the endogenous adenoviral gene that it replaces.

A second object of the invention is to describe recombinant adenoviral vectors that have restriction sites in the early region genes of the E3 region that encode the 6.7K and gp19K proteins.  
20 A third object of the invention is to describe recombinant adenoviral vectors that have restriction sites in the E3 region that encode the 10.4K, 11.6K, 14.5K, and 14.7K proteins.

A fourth object of the invention is to describe recombinant adenoviral vectors that have restriction sites in the E3 region that facilitate partial or total deletion of this region, or select genes contained therein, and compositions and methods for substituting foreign DNA therein.  
25

A fifth object of the invention is to describe methods for making recombinant adenoviral vectors that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select gene(s) contained therein.

A sixth object of the invention is to describe host cells containing recombinant adenoviral vectors that have a partial or total deletion of the E3 region, or select gene(s) contained therein.  
30

A seventh object of the invention is a description of recombinant adenoviral mutants that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select gene(s) contained therein.

An eighth object of the invention is a description of recombinant adenoviral mutants that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select genes contained therein, including virions; E3SV, E3SV +V, E3SV + B, and E3SV +V + B.  
35

A ninth object of the invention is a description of recombinant adenoviral mutants that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region wherein such mutants also have mutations elsewhere in the adenoviral genome, preferably in the E1A, E1B, and/or E4 regions.

5 A tenth object of the invention is a description of methods and compositions for diagnosing or treating disease, preferably diseases involving unwanted cell growth, including neoplasia, using recombinant adenoviral mutants that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select genes contained therein, wherein the adenoviral mutants have substituted in the E3 region genes that encode medically beneficial proteins. Preferred substituted genes  
10 include heterologous genes including negative selection genes, preferably cytosine deaminase, and thymidine kinase.

These and other objects of the present invention will become apparent to one of ordinary skill in the art upon reading the description of the various aspects of the invention in the following specification. The foregoing and other aspects of the present invention are explained in greater detail in the drawings,  
15 detailed description, and examples set forth below.

#### Brief Description of the Drawings

Figure 1 shows a map of the E3 region transcriptional unit of adenovirus type 5. The split arrows indicate the spliced structures of the mRNAs (open rectangles or solid lines represent Exons; dashed lines, Introns); the thickness of the arrow indicates the relative abundance. The shaded bars above the arrows indicate the E3 proteins, which are named on the basis of their molecular masses.  
20

Figure 2 shows the production of recombinant virus using pNB and Ad5 TP-DNA (m. u. stands for map units).

Figure 3 shows the production of recombinant virus using pSN with the desired mutation and Ad5 TP-DNA. The same strategy can also be used for the pG-based plasmids containing mutations.

25 Figure 4 shows the restriction map of the E3 region of the adenovirus E3SV

Figure 5 shows the restriction map of the E3 region of the adenovirus E3SV +V

Figure 6 shows the restriction map of the E3 region of the adenovirus E3SV +B

Figure 7 shows the restriction map of the E3 region of the adenovirus E3SV + V +B

Figure 8 shows A549 cells mock infected or infected with Ad5.

30 Figure 9 shows A549 cells infected with viruses Onyx 301, Onyx 302, Onyx 303, and Onyx 304.

Figure 10 shows western blot analysis of gp19k from cell lysates prepared from cells infected with viruses Onyx 301, Onyx 302, Onyx 303, and Onyx 304 at different times post infection.

35 Figure 11 shows a CD assay on cell lysates (0.5ug of protein) prepared from cells infected with viruses Onyx 301, Onyx 302, Onyx 303, Onyx 304, and Onyx 305 at different times post infection.

Figure 12 shows a CD assay on cell lysates prepared from cells infected with viruses Onyx 301, Onyx 302, Onyx 303, Onyx 304, and Onyx 305 at different times post infection using 0.6 ug protein/reaction.

5 Figure 13 shows the cytopathic effect of viruses Onyx 305 and Onyx 320 at different times post infection.

Figure 14 shows the cytopathic effect of virus Onyx 320 on cells that have, or have not had medium changes at certain times post infection.

Figure 15 shows the expression of CD at different times by cells infected with virus Onyx 305.

10 Figure 16 shows Western blots of the E3 proteins from A549 cells infected with Ad5. The numbers above each lane refer to times post-infection, p. i.

Figure 17 shows Western blots of E3 proteins from cells infected with Ad5 both in the absence and presence of araC, an inhibitor of DNA replication. The numbers above each lane refer to times post-infection, p. i.

15 Figure 18 shows a Western blot of the pVIII protein from the L4 region from cells infected with either Ad5 or Onyx 320, or mock-infected cells (M). The numbers above each lane refer to times post-infection, or p. i.

Figure 19 shows Western blots of the E3 proteins 11.6K, 14.5K, and 14.7K from cells infected with 2 CD containing viruses, Onyx 303 and Onyx 304. These levels can be compared to the levels of E3 proteins produced by Ad5 virus shown in figure 16.

20 Figure 20 shows a Western blot of 11.6K protein from cells infected with Ad5 or Onyx 305.

Figure 21 shows a Western blot of the 26 kD intracellular form of murine TNF (mTNF) synthesized by Onyx 320 infected cells. As controls, mock infected and Ad5 infected cells were also analyzed for the presence of mTNF.

25 Figure 22 shows a Western blot of 17 kD mTNF secreted into the culture medium from Onyx 320 infected cells. The medium was changed one hour before each indicated time point and then aliquots were taken at the indicated times post infection. 25  $\mu$ l of culture medium was run on a gel and blotted.

Figure 23 shows the results of an ELISA assay which measured the amounts of secreted mTNF present in the culture supernatant as described in figure 22. The results of 4 individual experiments are shown.

30 Figure 24 shows Western blots of the E3 proteins gp19K, 14.5K, and 14.7K from A549 cells infected with Onyx-320. As a control, two time points from an Ad5 infection are included. The numbers above each lane refer to times post-infection, p. i.

Figure 25 shows a Western blot of intracellular mTNF from cells infected with Onyx 320, and incubated in the presence or absence of araC.

Figure 26 shows Western blots of the L4 protein, pVIII, from cells infected with Ad5 or Onyx 304, 305, and 320 and incubated in the presence or absence of araC.

Figure 27 shows Western blots of the E3 protein gp19K from cells infected with either Ad5 or Onyx 320 and incubated in the presence or absence of araC.

5      Figure 28 shows pictures of A549 cells which have been infected with either Ad5 or Onyx 321 and photographed at the indicated times post-infection.

Figure 29 shows Western blots of mTNF, and the E3 proteins 14.5K and 14.7K from A549 cells infected with either Ad5 or Onyx 321. The results of mock (M) infected cells at 24 hours are also shown. The numbers above each lane refer to times post-infection.

10     Figure 30 shows Western blots of the E3 proteins gp19K and 11.6K from cells infected with Ad5 or Onyx 321. The numbers above each lane refer to times post-infection.

Figure 31 shows a Western blot of mTNF from cells infected with Onyx 321 incubated in the absence or presence of araC.

15     Figure 32 shows Western blots of the E3 proteins gp19K and 11.6K from cells infected with Onyx 321 either in the absence or presence of araC. The numbers above each lane refer to times post-infection.

#### Detailed Description of the Invention

All publications, including patents and patent applications, mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

#### Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures described below are those well known and commonly employed in the art.

Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and 30 various general references (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, organic synthetic chemistry, and pharmaceutical formulation described below are those well known and commonly employed in the art. Standard techniques are used for chemical 35 syntheses, chemical analyses, pharmaceutical formulation and delivery, and treatment of patients.

Those skilled in the art will also recognize publications that facilitate genetic engineering of the invention adenovirus to produce mutants in the E3 region. Such would include McGrory, W., J. et al., (1988) *Virology*, vol. 177, pp. 437-444 who describe insertion of DNA into the E1 region; Hanke, T., et al. (1990) *Virology*, vol. 177, pp. 437-444 and Bett, A. J. et al. (1993) *J. Virol.* vol. 67, pp. 5911-5921 5 who describe insertion of foreign DNA into the E3 region; and Bett, A. J. et al. (1994) *Proc. Natl. Acad. Sci.* vol. 91, pages 8802-8806, who describe insertion of DNA into the E1 and E3 regions. See also, Hermiston, T. et al., *Methods in Molecular Medicine: Adenovirus Methods and Protocols*, W.S.M. Wold, ed, Humana Press, 1999.

In the formulae representing selected specific embodiments of the present invention, the amino- 10 and carboxy-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. Thus, the N-terminal H<sub>2</sub><sup>+</sup> and C-terminal-O<sup>-</sup> at physiological pH are understood to be present though not necessarily specified and shown, either in specific examples or in generic formulas. In the polypeptide notation used herein, the left hand end of the molecule is the amino terminal end and the right hand end is the carboxy-terminal 15 end, in accordance with standard usage and convention. Of course, the basic and acid addition salts including those which are formed at non-physiological pH values are also included in the compounds of the invention. The amino acid residues described herein are preferably in the "L" isomeric form. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as a,a-distributed amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids 20 may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded residue where appropriate is represented by a three letter designation, corresponding to the trivial name of the conventional amino acid, in keeping with standard polypeptide nomenclature (described in *J. Biol. Chem.*, 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)). Free functional groups, including 25 those at the carboxy- or amino-terminus, referred to as noninterfering substituents, can also be modified by amidation, acylation or other substitution, which can, for example, change the solubility of the compounds without affecting their activity.

As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

30 The term "isolated protein" referred to herein means a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) is not associated with proteins found in nature, (2) is free of other proteins from the same source, e.g. free of human proteins, (3) is expressed by a cell from a different species, or (4) does not occur in nature.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an 35 object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in

an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "adenovirus" as referred to herein indicates over 40 adenoviral subtypes isolated from humans, and as many from other mammals and birds. See, Strauss, "Adenovirus infections in humans,"

5 in The Adenoviruses, Ginsberg, ed., Plenum Press, New York, NY, pp. 451-596 (1984). The term preferably applies to two human serotypes, Ad2 and Ad5.

The term "polynucleotide" as referred to herein means a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

10 The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages.

Oligonucleotides are a polynucleotide subset with 200 bases or fewer in length. Preferably oligonucleotides are 10 to 60 bases in length. Oligonucleotides are usually single stranded, e.g. for probes; although oligonucleotides may be double stranded, e.g. for use in the construction of a gene

15 mutant. Oligonucleotides of the invention can be either sense or antisense oligonucleotides. The term "naturally occurring nucleotides" referred to herein includes deoxyribonucleotides and ribonucleotides.

The term "modified nucleotides" referred to herein includes nucleotides with modified or substituted sugar groups and the like known in the art.

As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker,

20 e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., <sup>3</sup>H, <sup>14</sup>C, <sup>35</sup>S, <sup>125</sup>I,

25 <sup>131</sup>I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, b-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

30 The term "sequence homology" referred to herein describes the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are

35 usually used, 6 bases or less are preferred with 2 bases or less more preferred.

The term "selectively hybridize" referred to herein means to detectably and specifically bind. Polynucleotides, oligonucleotides and fragments of the invention selectively hybridize to nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective 5 hybridization conditions as known in the art and discussed herein. Generally, the nucleic acid sequence homology between the polynucleotides, oligonucleotides, and fragments of the invention and a nucleic acid sequence of interest will be at least 80%, and more typically with preferably increasing homologies of at least 85%, 90%, 95%, 99%, and 100%.

Two amino acid sequences are homologous if there is a partial or complete identity between 10 their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an 15 alignment score of more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., in *Atlas of Protein Sequence and Structure*, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN 20 program.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence 25 is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of 30 sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison: a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two 35 polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide

sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides. Sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window," as may be used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially

pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, and 99%. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single 5 macromolecular species.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity, and most preferably at least 99 percent sequence identity. Preferably, 10 residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids 15 having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamic-aspartic, and asparagine-glutamine.

20 The term "polypeptide fragment" or "peptide fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full-length cDNA sequence. Fragments typically 8-10 amino acids long, preferably at least 10-20 amino acids long, and even more preferably 20-70 amino acids long.

25 Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference.

The production of proteins from cloned genes by genetic engineering is well known. See, e.g. U.S. Patent Number 4,761,371 to Bell *et al.* at column 6, line 3 to column 9, line 65. The discussion 30 which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

DNA which encodes proteins that may be inserted into the adenoviral constructs of the instant invention in the E3 region can be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic 35 libraries from appropriate cells, or by combinations of these procedures, as illustrated below. Screening

of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from known gene sequence information. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays, as described in greater detail in the Examples below.

5 In the alternative, a gene sequence may be recovered by use of the polymerase chain reaction (PCR) procedure. See U.S. Patent Numbers 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

A vector is a replicable DNA construct. Preferred embodiment vectors described herein to realize the adenovirus E3 mutants are based on the pGEM vector series of Promega Corporation. Vectors are used either to amplify DNA encoding a desired protein and/or to express DNA which encodes the protein. An expression vector is a replicable DNA construct in which a DNA sequence encoding a protein of interest is operably linked to suitable control sequences capable of affecting the expression of the protein in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding a suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques.

DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, 30 contiguous and in reading frame. A preferred embodiment promoter of the instant invention in those instances where certain E3 region DNA is deleted and DNA substituted therein is a tissue specific promoter which is operably linked to a negative selection gene.

Suitable host cells include prokaryotes, yeast cells, or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example *Escherichia coli* (*E. coli*) or Bacilli. 35 Higher eukaryotic cells include established cell lines of mammalian origin as described below.

Exemplary host cells are DH5 $\alpha$ , *E. coli* W3110 (ATCC 27,325), *E. coli* B, *E. coli* X1776 (ATCC 31,537) and *E. coli* 294 (ATCC 31,446).

- A broad variety of suitable microbial vectors are available, and may have applications in constructing the instant adenoviral vectors. Generally, a microbial vector will contain an origin of replication recognized by the intended host, a promoter which will function in the host and a phenotypic selection gene such as a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement. Similar constructs will be manufactured for other hosts. *E. coli* is typically transformed using pBR322. See Bolivar *et al.*, *Gene* 2, 95 (1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.
- 10 Expression vectors should contain a promoter which is recognized by the host organism. This generally means a promoter obtained from the intended host. Promoters most commonly used in recombinant microbial expression vectors include the beta-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, *Nature* 275, 615 (1978); and Goeddel *et al.*, *Nucleic Acids Res.* 8, 4057 (1980) and EPO Application Publication Number 36,776) and the *tac* promoter (H. De Boer *et al.*, *Proc. Natl. Acad. Sci. USA* 80, 21 (1983)). While these are commonly used, other microbial promoters are suitable. Details concerning nucleotide sequences of many promoters have been published, enabling a skilled worker to operably ligate them to DNA in plasmid or viral vectors (Siebenlist *et al.*, *Cell* 20, 269, 1980)).

Cultures of cells derived from multicellular organisms are a desirable host for recombinant protein synthesis. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred. Propagation of such cells in cell culture has become a routine procedure. See *Tissue Culture*. Academic Press, Kruse and Paterson, editors (1973). Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and FL5.12, WI138, BHK, COS-7, CV, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral source (e.g. Polyoma, Adenovirus, VSV, or BPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources, including adenovirus. A variety of viral and mammalian constitutive promoter elements can be used. See, Mittal *et al.*, (1993) *Virus Research*, vol. 28, pp. 67-90. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. Patent Number 4,599,308. The early and

late promoters are useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. See Fiers *et al.*, *Nature* 273, 113 (1978).

#### Construction of Adenovirus E3 Mutants

Methods for the construction of adenoviral mutants are generally known in the art. See, Mittal, S. K., *Virus Res.*, 1993, vol. 28, pages 67-90; and Hermiston, T. et al., *Methods in Molecular Medicine: Adenovirus Methods and Protocols*, W.S.M. Wold, ed, Humana Press, 1999. Further, the adenovirus 5 genome is registered as Genbank accession #M73260, and the virus is available from the American Type Culture Collection, Rockville, Maryland, U. S. A., under accession number VR-5.

Generally, adenovirus vector construction involves an initial deletion or modification of a desired region of the adenoviral genome, preferably the Ad5 genome, in a plasmid cassette using standard techniques.

The adenoviral DNA, or a fragment thereof, present in pNB and which corresponds to the E3 region of the virus is subsequently cloned into another plasmid which may also be pGEM5zf+. For example, the SpeI-NdeI fragment corresponding to bases 27082-31089, of the Ad5 genome can be excised from pNB and cloned into the SpeI and NdeI sites in the multiple cloning site (MCS) of pGEM5zf+. This vector is termed pSN, and the adeno DNA present therein, bases 27082-31089, may be used to engineer the desired restriction sites into the E3 region to yield the appropriate E3 vectors, plasmids and viruses, discussed more below.

Certain of the materials and methods used to construct adenovirus mutants are described by Hanke, T., et. al. (1990) *Virology*, vol. 177, pages 437-444, and Bett, A. J., et. al., (993) *J. Virol.* vol. 67, pages 5911-5921, and in PCT/CA96/00375. Microbix Biosystems, Inc., located at 341 Bering Avenue, Toronto, Ontario Canada, sells many of the materials used to construct adenovirus mutants, and provides Product Information Sheets on how to make them. See also, Hermiston, T. et al., *Methods in Molecular Medicine: Adenovirus Methods and Protocols*, W.S. Wold, ed, Humana Press, 1999

It is noteworthy that while the instant invention is described in terms of adenovirus type 5, it may be practiced with other similar adenovirus serotypes. The general organization of the adenoviral genome is conserved among serotypes, and specific functions are similarly situated.

The mutations in the E3 region described herein may be incorporated into adenoviral mutants that have mutations outside the E3 region. Preferably such mutations would be in the E1B and/or E1A and/or the E4orf6 regions of the adenoviral genome. In the case of E1B mutations, the preferred mutations confer on adenovirus the ability to preferentially replicate in neoplastic cells compared to normal cells, wherein the neoplastic cells are functionally defective in the tumor suppressor, p53. Such mutations typically occur in the E1B region that encodes the 55kD protein. Defective p53 can arise in numerous ways, including a defect in those proteins that interact with p53; that is, a defect in the p53 pathway that renders p53 functionally inactive. See, U. S. Patent No. 5, 677, 178. Thus, the E3

mutations described herein could be combined with the E1B deletion in the adenovirus dl1520. This virus is described by Barker and Berk (1987) *Virology* 156: 107.

In the case of E1A mutations, the preferred mutations confer on adenovirus the ability to preferentially replicate in neoplastic cells compared to normal cells, wherein the neoplastic cells are functionally defective in the retinoblastoma tumor suppressor gene product, or p105 Rb. Such inactivating mutations in Ad5, typically occur in amino acids 30-85 in the E1A CR1 domain, or nucleotide positions 697-790, and/or the CR2 domain, amino acids 120-139, nucleotide positions 920-967, which are involved in binding the p105 Rb protein. Preferably, the CR3 domain of the adenoviral genome (spanning amino acids 150-188) remains and is expressed as a truncated p289R polypeptide and is functional in transactivation of adenoviral early genes. Defective pRb can arise in numerous ways, including a defect in those proteins that interact with pRb; that is, a defect in the pRb pathway that renders pRb functionally inactive. See, U. S. Patent No. 5, 677, 178. Thus, the E3 mutations described herein could be combined with the E1A deletion in the adenovirus Ad5 NT dl 1010.

Another aspect of the instant invention is the incorporation of heterologous genes into the E1B, E1A, or E4orf6 regions of an E3 mutant virus described herein. Thus, such viruses would contain heterologous genes in E3, and optionally in E1B, E1A or E4orf6. Examples of such heterologous genes, or fragments thereof that encode biologically active peptides, include those that encode immunomodulatory proteins, and prodrug activators (i.e. cytosine deaminase, thymidine kinase, U. S. Patent Nos. 5, 358, 866, and 5, 677, 178). Examples of the former would include interleukin 2, U.S. Patent Nos. 4,738, 927 or 5, 641, 665; interleukin 7, U. S. Patent Nos. 4, 965, 195 or 5, 328, 988; and interleukin 12, U. S. Patent No. 5,457, 038; tumor necrosis factor alpha, U. S. Patent Nos. 4, 677, 063 or 5, 773, 582; interferon gamma, U.S. Patent Nos. 4, 727, 138 or 4, 762, 791; or GM-CSF, U.S. Patent Nos. 5, 393, 870 or 5, 391, 485. Additional immunomodulatory proteins further include macrophage inflammatory proteins, including MIP-3, (See, Well, T. N. and Peitsch, MC. *J. Leukoc. Biol* vol 61 (5): pages 545-50,1997), and cell suicide, or apoptosis inducing proteins, including BAD and BAX. See, Yang, E., et al. *Cell*, vol 80, pages 285-291 (1995); and Sandeep, R., et al *Cell*, vol. 91, pages 231-241 (1997). Monocyte chemotactic protein (MCP-3 alpha) may also be used. A preferred embodiment of a heterologous gene is a chimeric gene consisting of a gene that encodes a protein that traverses cell membranes, for example, VP22 or TAT, fused to a gene that encodes a protein that is preferably toxic to cancer but not normal cells.

As mentioned above, the initial step in the construction of recombinant adenoviral vectors having novel restriction sites in the E3 region that facilitate partial or total deletion of the E3 region genes, or select genes contained therein, is to make mutations in the adenoviral genome in a plasmid cassette using well established techniques of molecular biology, or modifications these techniques, referred to herein. The following restrictions sites were engineered into the E3 region of adenovirus 5:

PacI, ClaI, PmeI, SwaI, BamHI, BstBI, SspI, NheI, and StuI and EcoRV. Their relative positions in the E3 region are shown in figures 4-7. The restriction sites were positioned so as not to knowingly disrupt critical splicing and polyadenylation signals. Another consideration was the coding sequence of proteins in the E3 region; in most cases, the mutations that were made to add the novel restriction sites did not 5 result in a change in the coding sequence; however, when amino acid changes were made, they were conservative in nature.

Thus, it is important to point out a key advantage of such adenoviruses that have inserted in the E3 region a heterologous gene or genes, which is that such gene(s) will preferably exhibit an expression pattern, both in terms of timing and degree of expression, similar to the endogenous adenoviral gene(s) 10 that it replaces.

The adenoviral vectors of the instant invention can also incorporate a tissue specific promoter in a part of the E3 region that has been deleted that will drive the expression of another gene, preferably a negative selection gene. An example of a tissue specific promoter includes prostate specific antigen promoter. See, PCT/US95/14461. Examples of certain negative selection genes include cytosine 15 deaminase, and thymidine kinase. Regarding cytosine deaminase, see, U. S. Patent Nos. 5, 358, 866, and 5, 677,178.

For example, a HSV *tk* gene cassette may be operably linked downstream of an E3 promoter. Frequently, it is desirable to delete a nonessential portion (i.e., for viral replication and packaging) of the adenoviral genome to accommodate the negative selection cassette; thus a substantial portion of the E3 20 gene region may be deleted and replaced with a negative selection cassette such as an HSV *tk* gene operably linked to either an E3 promoter, or a tissue specific promoter (and enhancer) or other suitable promoter/enhancer. Alternatively, a negative selection gene may be operably linked to an adenovirus late region promoter to afford efficient expression of the negative selection gene product in cells expressing a replication phenotype characterized by transcription from late gene promoters.

25 Expression of the HSV *tk* gene in a cell is not directly toxic to the cell unless the cell is exposed to a negative selection agent such as gancyclovir or FIAU. Infected cells expressing a replication phenotype wherein a negative selection gene is substantially expressed may produce essentially no additional cytotoxicity until the negative selection agent (e.g., gancyclovir) is administered in an effective selective dosage, at which time the infected cells expressing the *tk* gene will be selectively 30 ablated; thus negative selection can be used for enhanced cytopathic killing and/or to damp out further viral replication by killing cells exhibiting a replicative phenotype.

A preferred embodiment is an HSV *tk* gene cassette (Zjilstra et al. (1989) *Nature* 342:435; Mansour et al. (1988) *Nature* 336: 348; Johnson et al. (1989) *Science* 245: 1234; Adair et al. (1989) *Proc. Natl. Acad. Sci (U.S.A.)* 86: 4574; Capecchi, M. (1989) *Science* 244:1288, incorporated herein by reference) operably linked to an appropriate promoter and/or enhancer with a polyadenylation site to 35

form a *tk* expression cassette. The *tk* expression cassette (or other negative selection expression cassette) is inserted into the adenoviral genome, for example, as a replacement for a substantial deletion of the E3 region.

The adenoviral vectors of the instant invention that encode a desired protein can be used for transformation of a suitable mammalian host cell. Transformation can be by any known method for introducing polynucleotides into a host cell, including, for example packaging the polynucleotide in a virus and transducing a host cell with the virus or by transfection procedures known in the art, as exemplified by U.S. Patent Nos. 4,399,216, 4,912,040, 4,740,461, and 4,959,455. The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

#### Therapeutic Methods

Therapy of disease, preferably neoplastic disease, may be afforded by administering to a patient a composition comprising adenoviruses of the invention, and further comprising a negative selection gene. Examples of the latter would include cytosine deaminase and thymidine kinase.

Various human neoplasms may be treated with the invention adenoviral constructs, particularly in those instances where the E3 region of the virus encodes a protein useful for gene therapy of disease. An example would be a cytokine, preferably an interleukin. For example, but not by way of limitation, a human patient or nonhuman mammal having a bronchogenic carcinoma, nasopharyngeal carcinoma, laryngeal carcinoma, small cell and non-small cell lung carcinoma, lung adenocarcinoma, hepatocarcinoma, pancreatic carcinoma, bladder carcinoma, colon carcinoma, breast carcinoma, cervical carcinoma, ovarian carcinoma, or lymphocytic leukemias may be treated by administering an effective antineoplastic dosage of an appropriate adenovirus. Suspensions of infectious adenovirus particles may be applied to neoplastic tissue by various routes, including intravenous, intraperitoneal, intramuscular, subdermal, and topical. An adenovirus suspension containing about  $10^3$  to  $10^{12}$  or more virion particles per ml may be inhaled as a mist (e.g., for pulmonary delivery to treat bronchogenic carcinoma, small-cell lung carcinoma, non-small cell lung carcinoma, lung adenocarcinoma, or laryngeal cancer) or swabbed directly on a tumor site for treating a tumor (e.g., bronchogenic carcinoma, nasopharyngeal carcinoma, laryngeal carcinoma, cervical carcinoma) or may be administered by infusion (e.g., into the peritoneal cavity for treating ovarian cancer, into the portal vein for treating hepatocarcinoma or liver metastases from other non-hepatic primary tumors) or other suitable route, including direct injection into a tumor mass (e.g., a breast tumor), enema (e.g., colon cancer), or catheter (e.g., bladder cancer).

The invention adenovirus mutants may be further evaluated by their capacity to reduce tumorigenesis or neoplastic cell burden in *nu/nu* mice harboring a transplant of neoplastic cells, as compared to untreated mice harboring an equivalent transplant of the neoplastic cells.

Adenoviral therapy using the instant invention E3 viruses may be combined with other antineoplastic protocols, such as conventional chemotherapy. Also, in the event that the instant E3 adenoviral vectors, or viruses elicit an immune response that dampens their effect in a host animal, they can be administered with an appropriate immunosuppressive drug.

#### Propagation of Mutant Adenovirus

Adenoviral mutants of the invention typically are propagated as viral stocks in a cell line (e.g., 10 the 293 cell line ATCC # CRL 1573, American Type Culture Collection, Rockville, MD; Graham et al. (1977) *J. Gen. Virol.* **36**: 59, or A549 cells) that can provide certain desired viral functions, if needed, in trans to support replication and formation of infectious mutant virions.

#### Formulations

15 Adenovirus E3 mutants may be formulated for therapeutic and diagnostic administration to a patient. For therapeutic or prophylactic uses, a sterile composition containing a pharmacologically effective dosage of one or more species of adenovirus mutant is administered to a human patient or veterinary non-human patient for treatment, for example, of a neoplastic condition. Generally, the composition will comprise about  $10^3$  to  $10^{15}$  or more adenovirus particles in an aqueous suspension. A pharmaceutically acceptable carrier or excipient is often employed in such sterile compositions. A 20 variety of aqueous solutions can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter other than the desired adenoviral virions. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, 25 sodium lactate, etc. Excipients which enhance infection of cells by adenovirus may be included.

Adenoviruses of the invention, or the DNA contained therein, may be delivered to neoplastic cells by liposome or immunoliposome delivery; such delivery may be selectively targeted to neoplastic cells on the basis of a cell surface property present on the neoplastic cell population (e.g., the presence of a cell surface protein which binds an immunoglobulin in an immunoliposome). Typically, an aqueous 30 suspension containing the virions are encapsulated in liposomes or immunoliposomes. For example, a suspension of adenovirus virions can be encapsulated in micelles to form immunoliposomes by conventional methods (U.S. Patent 5,043,164. U.S. Patent 4,957,735. U.S. Patent 4,925,661; Connor and Huang (1985) *J. Cell Biol.* **101**: 582; Lasic DD (1992) *Nature* **355**: 279; *Novel Drug Delivery* (eds. Prescott LF and Nimmo WS: Wiley. New York. 1989); Reddy et al. (1992) *J. Immunol.* **148**: page 35 1585). Immunoliposomes comprising an antibody that binds specifically to a cancer cell antigen (e.g.,

CALLA, CEA) present on the cancer cells of the individual may be used to target virions, or virion DNA to those cells.

The compositions containing the present adenoviruses or cocktails thereof can be administered for prophylactic and/or therapeutic treatments of neoplastic disease. In therapeutic application, compositions are administered to a patient already affected by the particular neoplastic disease, in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious dose." Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, and the route of administration.

In prophylactic applications, compositions containing the invention adenoviruses, or cocktails thereof, are administered to a patient not presently in a neoplastic disease state to enhance the patient's resistance to recurrence of a neoplasm or to prolong remission time. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antineoplastic adenoviruses of this invention sufficient to effectively treat the patient.

Antineoplastic adenoviral therapy of the present invention may be combined with other antineoplastic protocols, such as conventional chemotherapy.

#### Uses of the Invention

It will be apparent, based on the discussion above, that the adenoviral vectors/viruses described herein have multiple uses including applications in gene therapy. For example, in one embodiment of the invention, a gene that encodes a medically useful protein may be cloned into the E3 region of the instant invention virions, and the virions used directly in gene therapy protocols to treat disease. In another embodiment of the invention, discussed above, such E3 mutant virions may also have deletions/mutations in other regions of the adenoviral genome, including in the E1B region and have substituted therefore a gene with desirable properties. In either the E3 or E1B or other regions, such genes might encode cytokines, including the interleukins, cell cycle regulatory proteins, including p16, ras, or proteins that induce cellular suicide or apoptosis, prodrug activators, including cytosine deaminase or thymidine kinase. Further, tumor necrosis factor alpha, interferon gamma, and mip-3 may be utilized. Additionally, genes that encode anti-angiogenic factors could be used.

The instant adenoviral vectors may also be used to express proteins that are useful immunogens, or as a vaccine, and to transform cells which do not ordinarily express a particular protein to thereafter express this protein. Cells expressing these molecules are useful as intermediates for making cell

membrane preparations useful for binding assays, which are in turn useful for drug screening.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

5

### Example 1

#### General Methods and Working Vectors

Methods for the construction and propagation of human adenovirus vectors are known in the art and will be understood to be applied in the Example presented below by the skilled practitioner of the art. Such would include the work of Hitt, M., et al Construction and propagation of human adenovirus vectors. In: Cell Biology: a Laboratory Handbook; J. Celis (Ed), Academic Press, N. Y. (1996); Graham, F. L. and Prevec, L. Adenovirus based expression vectors and recombinant vaccines. In: Vaccines: New Approaches to Immunological Problems. R. W. Ellis (ed) Butterworth. Pp. 363-390; and Graham, F. L. and Prevec, L. Manipulation of adenovirus vectors. In: Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Techniques. E. J. Murray and J. M. Walker (eds) Humana Press Inc., Clifton, N. J. pp 109-128, 1991. The materials and methods described in these articles were used below. See also, Hermiston, T. et al., Methods in Molecular Medicine: Adenovirus Methods and Protocols, W.S.M Wold, ed, Humana Press, 1999.

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#### Adenoviral Vectors:

Vectors based on pGEM (Promega Corp.) were modified and used to clone, subclone, and 20 mutagenize the appropriate E3 region of Ad5. This took advantage of the Ad5 existing restriction sites in and outside the E3 region, and these are shown in Table 1.

Table 1

Restriction sites present in Ad5

NdeI	19549 and 31089
SpeI	27082
EcoRI	27295 and 30049
SunI	28390
EcoRV	27295
KpnI	28787
MunI	29355
NotI	29510
XhoI	29791
HpaI	30569

The vector designated pSN was subcloned by inserting the fragment from Ad5 SpeI (27082) to NdeI (31089) into the SpeI and NdeI sites in the multiple cloning site (MCS) in pGEMSzf.

The vector pGEM5 was further modified because it possesses 3 SspI sites (2199, 2384, 2408); SspI is one of the engineered sites in the E3 shuttle vector. By deleting the SspI sites in the vector, 5 inserting genes into the E3 SspI site would be facilitated since it would not involve partial restriction digests. To delete the vector sites, the plasmid pGEM5 was cut with SspI and EcoRV (present in the MCS at base 51) and religated. Unfortunately, the SspI site at 2199 was not deleted and found in the resultant altered vectors. So the vector contains the deletion from SspI at 2384 to EcoRV at 51. The presence of this extra SspI site still requires partial restriction digestion when utilizing SspI in the E3 10 region, although the isolation of the correct fragment is simplified by the deletion of two of those sites. Note also that there is an SspI site in the E3 region at 30172; this resides in the same region that would be excised when the engineered SspI site is to be used. Thus, it is of no consequence. This altered pGEM vector was used as the vector to insert the SpeI to NdeI region of Ad5, and for subsequent manipulations in the E3 regions and is indicated by pG.

15

#### Example 2

##### Construction of E3 Shuttle Vectors

Using the above vectors, the following restrictions sites were engineered into the E3 region of adenovirus 5: PacI, Clal, Pmel, SwaI, BamHI, BstBI, SspI, NheI, and StuI and EcoRV. Their relative positions in the E3 region are shown in figure 7. The restriction sites were carefully positioned so as to 20 not knowingly disrupt, or minimally disrupt, critical splicing and polyadenylation signals (see, figure 1). Also considered was the coding sequence of proteins; in most cases, the coded amino acid was not changed, and when changes had to be made, they were conservative.

Because of the position of the engineered sites, some mutations had to be performed sequentially. All of the oligonucleotide sequences used for mutagenesis and the exact location (position 25 number in Ad5) are listed in the tables. All mutations were confirmed by restriction digests and all constructs were sequenced. Table 2 summarizes the restriction sites that were added to the E3 region of adenovirus 5.

**Table 2**

Restriction sites added to Ad5 E3 region. Numbers refer to the Ad5 genome.

PacI	28497
NheI	28532
PmeI	29310
BstBI	29484
StuI	29718
EcoRV*	29781
ClaI	29862
SspI	30377
BamHI**	30467
Swal	30830

\* The start codon for 10.4K was altered with this mutation

5    \*\*The start codon for 14.7K was altered with this mutation

The sites PacI and ClaI were generated simultaneously by using mutant oligonucleotide PacC plus PacNC and ClaC plus ClaNC, respectively, using the Transformer Site-Directed Mutagenesis kit (Clontech #K1600-1) as directed by the manufacturer. The sites PmeI and Swal were constructed separately using PmeC plus PmeNC and SwaC plus SwaNC, respectively, using the QuickChange Site-Directed Mutagenesis kit (Stratagene #200518) exactly as described by the manufacturer. The PmeI site was cloned into the PacI/ClaI-containing plasmid by inserting the PmeI-containing KpnI-XhoI (natural Ad5 sites) fragment into this plasmid. To this construct, the Swal site was inserted using the HpaI to NdeI fragment. This resulting construct was called pG-PPCS and used for the next round of mutagenesis.

15    **BamHI**

All of the following mutations were created by PCR-based mutagenesis (See, Nucleic Acids Research 17:5404; 1989, and U.S. Patent Numbers 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis) using PfU polymerase, a high fidelity enzyme (Stratagene). All fragments generated by PCR were subsequently sequenced and determined to be error-free. This procedure employs two sequential PCR reactions and cutting the final product for insertion into a desired plasmid. Briefly, the technique employs two PCRs and three primers, one containing the mutation of interest. In the first reaction, the template and the two primers are used with one of the primers having the desired mutation. In the second reaction, the amplified product from the first reaction is used as a primer along with a third primer. The final product is digested and purified for insertion into a desired plasmid using standard techniques.

The BamHI site was generated with the oligonucleotides BamC and SwaNC in the first PCR and this product with PmeC in the second PCR, using the above-described plasmid pG-PPCS as the template. This fragment (the product of the second PCR) was digested with Pmel and Swal to insert into pG-PPCS and this was termed pG-PPCS +B. It should be noted that this mutation also changes the 5 14.7K start codon to prevent premature initiation for any inserted gene at this site and that only two of the final four versions of the E3 shuttle vectors have the BamHI site. Vectors with this BamHI site are used when expression of 14.7K is not desired, or when 14.7 is replaced with a foreign gene.

#### BstBI, NheI, and StuI

The BstBI site was created using BstBC and SwaNC in the first PCR and PmeC plus the first 10 product in the second PCR. The template used was pG-PPCS. The second PCR product was digested with MunI and Swal and inserted into pG-PPCS, resulting in pG-PPBCS. To make a version of this vector that contained the BamHI site, the fragment from Clal to Hpal with this site was inserted to make pG-PPBCS+B.

The NheI site was made with NheC and PmeNC in the first PCR and SunC and the first product 15 in the second PCR, using pG-PPBCS+/- B as the templates in separate reactions. This fragment was digested with PacI and Pmel and inserted into the two versions of the above described constructs, pG-PPBCS+/-B. This construct was called pG-PNPBCS+/-B. The StuI site was added by using StuC and SwaNC in the first reaction and PmeC and the first product in the second reaction, with pG-PPBCS+/-B as templates. The fragment was digested with MunI and Swal and inserted into the two versions on the 20 plasmid described in the previous paragraph (- or + BamHI) and these were called pG-PPBSCS or pG-PPBSCS+B, respectively. The new StuI and NheI sites were added together by digesting the two plasmids with PacI and Pmel, and inserting the fragment which contained the NheI site (from pG-PNPBCS) into the StuI-containing plasmid (pG-PPBSCS). This was done in plasmids which did or did not have the BamHI site and the resulting plasmids were called pG-PNPBSCS+B and pG-PNPBSCS, 25 respectively.

#### SspI and EcoRV

The last two mutations were made using both the above-described plasmids as templates, pG-PNPBSCS and pG-PNPBSCS+B. The SspI site was created with SspC and HpaNC primers in the first PCR and NheC and the product of the first PCR in the second PCR. This fragment was digested with 30 Xhol and Hpal to insert into the parental plasmid, with or without the BamHI site. The EcoRV site was made using the EcoRVC and HpaNC primers in the first PCR and NheC and the first product in the second PCR. This fragment was cut with Xhol and Hpal to insert into the plasmids described in the above paragraph. The SspI and EcoRV sites were added together by cutting both BamHI-containing or BamHI-absent plasmids with Pmel and Clal and inserting the EcoRV-containing fragment into the 35 parental plasmids. Note the EcoRV site also changes the start codon for 10.4K to prevent premature

initiation of genes inserted into the *Cla*I site at their 5' end. The *Cla*I site will be used, instead of the EcoRV site, for insertion of genes since the region with the EcoRV site is also involved in splicing. Deletion of this region might disrupt this event.

These final versions of the E3 shuttle vectors are depicted in Figs. 4 through 7. These are called 5 pGE3SV, pGE3SV+V, pGE3SV+B, and pGE3SV+V+B, the differences being the presence or absence of the EcoRV and BamHI sites. These plasmids are on deposit with the American Type Culture Collection, accession numbers XXX, XXX, XXX, and XXX, respectively.

These shuttle vectors are used for construction of all subsequent plasmids, be they insertion of foreign genes or deletion of the Ad5 E3 genes.

10 The oligonucleotides that were used to mutagenize the desired E3 region are shown in Table 3.

**Table 3:**

Sequence of oligonucleotides used to mutagenize the E3 region of Adenovirus 5:

SunC	CCTCTCCGAGCTCAGCTACTCCATCAG
PacC	GGAGGTGAGCTTA <u>ATT</u> AACCCTTAGGG
PacNC	CCCTAAGGG <u>TTA</u> TTAACGCTCACCTCC
EcoRVC	GATTAAATGAGA <u>T</u> ATCATTCCCTCGAG
HpaNC	GGCGGTGTCCGGTGGTATTACTGTCG
NheC	GGGTATTAGGCCAAAGGCGCAGCT <u>AGCGT</u> GGGG
StuC	CCCAAACAATGA <u>AGGC</u> CTCCATAGATTGG
SspC	CAGCTACT <u>TTA</u> AT <u>ATT</u> ACAGGAGGAG
BstBC	GCGACCCACC <u>CTT</u> CGAACAGAGATGACCAAC
BamC	GGAGAC <u>G</u> ACTGACACC <u>CTGG</u> AT <u>CC</u> CAGAAATGG
ClaC	CACATCG <u>AT</u> GTAGACTGC
ClaNC	GCAGTCTAC <u>AT</u> CGATGTG
PmeC	TAGAATAG <u>GG</u> <u>TT</u> AAACCCCCGG
PmeNC	CCGGGGGG <u>TT</u> AA <u>AC</u> CC <u>CT</u> ATTCTA
SwaC	CTCAAAGAT <u>CTT</u> T <u>AT</u> CC <u>AT</u> TT <u>AA</u> <u>AT</u> AAA
SwaNC	TTTATT <u>TT</u> AA <u>AT</u> GGAATAAGAT <u>CTT</u> GAG
CD-PacC	GTGAGCTTA <u>ATT</u> A <u>AGG</u> CTAG <u>CA</u> AT <u>GT</u> CGA <u>AA</u> ACGC
CD-SwaNC	GTGAGC <u>AT</u> TT <u>AA</u> AT <u>CA</u> GT <u>CG</u> TT <u>CA</u> AC <u>GT</u> TT <u>GT</u> A <u>AT</u> C

- 5 All sequences are written 5' to 3'. All changed bases are underlined. Inserted bases are in bold.

### Example 3

#### Construction of Virus Controls and Optional Plasmids for Virus Production

For controls, each of the E3 genes was deleted using the engineered sites. To do this, the shuttle plasmids were cut with the following pairs of enzymes, filled in using T4 DNA polymerase, and 10 religated: PacI and PmeI, SunI and MunI, NheI and PmeI, BstBI and StuI (all in pG-E3SV), ClaI and SwaI (in pG-E3SV+V), ClaI and SspI (in pG-E3SV+V), and BamHI and SwaI (in pG-E3SV+B).

In addition to the pG plasmids referred to above that were used to generate the invention viruses, Figure 2 shows another plasmid that could also be used, termed pNB. The pNB has two SpeI sites: one in the Ad5 insert and one in the pGEMS MCS. The fragment from pNB which contained a portion of the 15 MCS and NdeI 19549 to SpeI 27082 was inserted into the SpeI-cut pG-PPCS plasmid. The orientation was confirmed to be correct, and the resulting plasmid termed pNB-PPCS. All of the final versions of the E3 shuttle vectors were cloned into this plasmid by inserting the PacI to SwaI region of the pG

plasmids into the larger pNB-PPCS. The resulting plasmids were designated pNB-E3SV, pNB-E3SV+V, pNB-E3SV+B, and pNB-E3SV+V+B, and could be used to produce viruses similar to those produced using the pG family.

#### Example 4

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##### Construction of CD Plasmids

To test the shuttle vector system for its therapeutic use, the *E. coli* gene cytosine deaminase (CD) was used because of its prodrug capabilities. CD was obtained from ATCC (#40999, plasmid pCD2) and the CD gene was amplified from this plasmid as follows. It should also be noted that the CD gene contains an NdeI restriction site; because we intended to use this particular enzyme to cut NdeI sites in the shuttle vectors, it was necessary to remove the NdeI site in the CD gene by PCR mutagenesis. This technique is the same one that was used to engineer in the new restriction sites into the E3 region, using the high-fidelity Pfu polymerase. Table 4 shows the oligonucleotides used to amplify the CD gene.

Briefly, a conservative mutation was made in the NdeI site, changing the base T to a C. The primers for the first PCR reaction were CD-NdeC and SwaCDNC. The plasmid pCD2 was used as a template. This product, along with the same template and primer CD-PacC, were used for the second PCR reaction. This accomplished two goals: it altered the NdeI site and added restriction sites PacI and Swal to the 5' and 3' ends, respectively. This final PCR product was cut with PacI and Swal; the shuttle vector, pGE3SV was also digested with PacI and Swal. The fragments were gel purified using the Qiagen gel extraction kit and then ligated together using NEB T4 DNA Ligase. The *E. coli* strain XL-1 was transformed with the ligation mix, plated on ampicillin-containing plates for selection, and colonies were picked and cultured. The DNA was isolated and then screened by restriction digest to check for correct insertion and deletion. The clones which appeared correct were then sequenced throughout the entire CD gene and surrounding vector to verify that no unwanted mutations had taken place. This correct and verified clone was called pG-CDPacSwa and used in subsequent PCR amplifications where the CD gene was amplified for insertion into other regions. It should be noted that the CD gene contains a bacterial start codon, GTG. In all 5' primers, the start codon was included and changed to the eukaryotic codon, ATG.

The other CD-containing vectors were created by designing the appropriate primers which possess the desired restriction site at the 5' or 3' end of the gene; the 5' primer always containing the ATG start codon. The CD gene was inserted into E3 regions using the following restrictions sites: BstBI to StuI, NheI to MunI, NheI to PmeI, PacI to PmeI, SmaI to MunI, Clal to Swal, and BamHI to Swal. The plasmids were named: pG-CDBstStu, pG-CDNheMun, pG-CDNhePme, pG-CDPacPme, pG-CDSunMun, pG-CDClaswa, pG-CDBamSwa, respectively. All primers used are listed in Table 3 and the template was always the confirmed plasmid pG-CDPacSwa. The CD gene in the Clal to Swal region was inserted into the pGE3SV+V plasmid; the CD gene in the BamHI to Swal region was inserted in the

E3SV+B plasmid. All insertions were sequenced completely to ensure that no unwanted mutations had taken place and that the CD gene was inserted correctly.

It should be noted that there are three choices for 5' insertion site and 2 choices for 3' site that can be used for inserting genes into the 6.7-gp19K region. These are SunI, PacI, and NheI for the 5' end, and PmeI and MunI for the 3'end. The PacI and SunI sites overlap the y-leader, an important sequence for translation of late gene products. Disruption of this sequence may abrogate its effect for certain applications. Therefore, another site, NheI was inserted which does not overlap the y-leader. If no adverse affect is seen, then the SunI to MunI sites, naturally present in Ad5, may be useful since it allows for a greater cloning capacity.

Several points are predicted from the viruses that result from the above E3 insertions. First, the construct that remove portions of the y-leader, as in pG-CDSunMun, may cause an adverse effect on the course of the infection, as discussed above. This may also be true of genes inserted into the PacI site, although less of the y-leader is deleted. Another prediction is that inserts into the 11.6K region, as in pG-CDBstStu, may result in a greatly attenuated infection. As has been published, deletion of the 11.6K protein (ADP or adenovirus Death Protein) does not allow the infected cells to lyse at the proper time, compared to wild type infection. In this case the cell continues to metabolize, the virus production per cell is higher, and the cell basically becomes a factory for producing the foreign gene. Also, since ADP is synthesized in large quantities using the major late promoter during the late phase of infection, a foreign gene inserted into this region would be expected to have the same expression characteristics. The results obtained with these viruses will be discussed below.

#### Example 5

##### Construction of TNF Plasmids

The plasmid containing the murine tumor necrosis factor (mTNF) gene was obtained from ATCC (#63169). This sequence contains the entire mTNF gene including the coding region for the prosequence. The mTNF gene was amplified from this plasmid by PCR (Table 4) and gel purified. The vector pGE3SV was cut with BstBI and StuI and gel-purified. At the same time, another vector, pG-E3SV+V was cut with Clal and SwaI and gel purified. The purified PCR product was readily inserted into each of these vectors due to compatible ends. These constructs were called pG-mTNFBstStu and pG-mTNFClaSwa, respectively.

Using the ATCC plasmid as a template once again, mTNF gene was amplified by PCR. The plasmid pG-E3SV+B and the PCR product were cut with BamHI and SwaI, gel purified, and ligated together. This construct was called pG-mTNFBamSwa. All constructs were sequenced extensively to check for unwanted mutations.

**Example 6****Construction of CD and TNF Viruses**

To build the above described CD constructs into the Ad5 genome, Ad5 TP-DNA was used. For 5 the mTNF containing viruses, BstLink TP-DNA was used since it offers certain advantages. The plasmid construction for this BstLink is described in Example 11. Note that all transfections were performed on 6 cm dishes and in duplicate; the quantities described here are per each 6 cm dish.

**Methods:** the viruses E3-CD-PacPme (Onyx 301), E3-CD-NhePme (Onyx 302), E3-CD-SunMun (Onyx 303), E3-CD-NheMun (Onyx 304), E3-CD-BstStu (Onyx 305), and E3-mTNF-BstStu 10 (Onyx 320) were made as follows: first, 0.5 micrograms of TP-DNA (Ad5 for CD viruses and BstLink for mTNF viruses) and ten micrograms of plasmid were cut with EcoRI (20 units) at 37 degrees for 5 hours. (An overabundance of plasmid DNA was used to allow approximately five micrograms of the actual insert DNA per transfection) At this point, the TP-DNA was left to digest at room temperature overnight while the cut plasmids were run on a 1% agarose gel overnight. The inserts were gel-purified 15 using the Qiagen gel extraction kit. Ligation reactions consisted of the cut TP-DNA and the purified fragments with 10 units of high concentration T4 DNA Ligase (Boehringer Mannheim) overnight at 16 degrees. This reaction mixture was used directly for transfection.

Homologous recombination was used to generate the viruses: CDPacSwa, mTNFClaSwa, 20 CDBamSwa, mTNFBamSwa, because these mutations in the E3 region lie outside of the EcoRI restrictions sites. The quantities of DNA and TP-DNA are the same as above. The TP-DNA BstLink was cut with BstBI for the transfection. The CD-containing plasmids were cut with SpeI and NdeI and the mTNF-containing plasmids were cut with PstI and NdeI. The fragments were gel-purified and eluted in water. For transfections, the cut TP-DNA and the isolated fragments were mixed together and used without any further manipulations.

**Transfection Procedures:** For transfections, A549 cells were plated onto 6 cm dishes the preceding day so that they would be approximately 70 to 80% confluent the day of the transfection. To 25 transfect these cells, 2 solutions were made and subsequently mixed. Solution A contained the ligation mixture and 300 ul of OptiMEM (Life Technologies) per 6 cm dish. Solution B contained 300 ul of OptiMEM and 13 microliters of Lipofectamine (Life Technologies). These two solutions were added 30 together, mixed gently, and allowed to incubate at room temperature for 30 to 45 minutes. Near the end of this incubation time, the cells were washed with warm OptiMEM and 2.4 ml of OptiMEM was added to each of the mixtures. This final 3 ml mix was then added directly to the washed cell monolayer and incubated at 37 degrees for 5 hours. Then 3 ml of DME containing 20% FBS was added to each dish without removing the transfection mix, bringing the final serum concentration to 10%. This was allowed 35 to incubate at 37 °C overnight. The cells were overlaid with 8 ml of DME/ 2% FBS/1.0% agar noble

(Difco). Five days after this overlay, another overlay (5 ml) was added which also contained 0.3 % neutral red (Life technologies) to help visualize the plaques.

Propagation and confirmation of virus mutants: As plaques appeared (10 to 20 days after transfection), they were isolated as agar plugs using a sterile Pasteur pipette. To propagate the virus present in the agar plugs, 3.5 cm plates were seeded with A549 cells in DME/10% FBS on the previous day. The day of the infection, the medium was changed to DME/2%FBS and the isolated plaques were added to the cells. The infections were checked daily for CPE (cytopathic effect, where the cells become rounded up and detach from the plate as a result of the virus infection), which usually occurred 3 to 5 days after infection. The entire medium and cells were collected and frozen at -20 degrees. To check for a virus mutation, 200 microliters of the cell and medium mix was used to isolate the viral DNA (along with cellular DNA) using the Qiagen Blood kit. This purified DNA was checked by PCR using primers which corresponded to the CD gene itself or the flanking E3 region. Once the PCR of the recombinant virus DNA was shown to produce a correct size fragment, further characterization included cutting the PCR fragments with restriction enzymes for patterns unique to CD or mTNF and also by sequencing the PCR product. Hirt analysis was also performed to confirm that the correct virus was obtained.

The correct viruses were expanded by infecting a T150 of A549 cells with 500 ul of the viral suspension obtained from the 3.5 cm dish. This was allowed to proceed to full CPE (when over 75% of the cells are no longer attached to the flask surface), which occurred in approximately 3 days. Then 7.5 ml of this cell and medium mixture was used to infect a 3-liter spinner of KB cells, and the virus produced was purified by CsCl-banding. Plaque assays were performed to determine the infectious particles per unit volume.

The viruses were named at this point in such a way to make it obvious to tell which insert had been added and where the insert was placed. Numbers for ease of reference were also assigned to each virus, and these appear in parenthesis. Their names are E3-CD-PacPme (Onyx 301), E3-CD-NhePme (Onyx 302), E3-CD-SunMun (Onyx 303), E3-CD-NheMun (Onyx 304), E3-CD-BstStu (Onyx 305), E3-mTNF-BstStu (Onyx 320), and E3-mTNF-ClaSwa (Onyx 321).

CD assay: To assay for cytosine deaminase (CD) activity, the reaction was performed similar to that as described in Rogulski et al 1997. Briefly, A549 cells were seeded into 10 cm plates so that they were about 70 to 80% confluent on the day of infection (about 2 to 4 million cells per plate). The cells were infected at an MOI (multiplicity of infection) of 10 pfu (plaque forming units) per cell for each of the E3-CD viruses. Ad5 and mock infected cells were included as controls. For the infection, the proper volume of virus was suspended in 2 ml of DME per 10 cm plate and then added to the cell monolayer. After one hour, 8 ml of DME/2% FBS medium was added to each plate. At various times post-infection (4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 120 hours), the cells were rinsed, 1ml of cold PBS was added, cells

were scraped (using disposable cell scrapers) and pelleted into 1.5 ml eppendorf tubes. All PBS was removed and the cell pellets were flash-frozen in dry ice/ethanol and stored at -80<sup>0</sup>C degrees. 200 ul of assay buffer (100 mM Tris/HCl (pH 8.0) 1 mM EDTA, 1mM B-mercaptoethanol) was added to each pellet and the cells were lysed by 4 freeze/thaw cycles. The lysates were cleared by centrifugation at full speed for 5 minutes at 4<sup>0</sup>C. The quantity of protein was determined by a Bradford assay using Bio-rad reagents. For the enzyme assay, either 5 micrograms or 0.6 micrograms of protein from each sample were used. along with 2.5 mM [2-14C]-cytosine (1 uCurie: 5 ul; Moravek Biochemicals, #MC131) and assay buffer to bring the reaction volume to 10 ul. The reaction was allowed to proceed for one hour at 37<sup>0</sup>C. To quench the reaction, 10 ul of cold cytosine/uracil (0.4 mg/ml each) was added. Ten ul from each sample was spotted onto a thin layer chromatography plate (Baker #7009-04) and then placed in an equilibrated tank with 1-butanol-water (86%/14%). After allowing the solvent front to approach the top of the plate (about 2 hours). the plate was allowed to dry and exposed to film. This autoradiogram was then scanned for the figures.

Murine TNF alpha assay: The A549 cells were plated onto 6 cm plates so that they would be approximately 80% confluent for the infection. The infection was performed as described above at an M.O.I. of 10. At various time points, the medium was removed and replaced with 3 ml of fresh DME/2% FBS. This was incubated at 37<sup>0</sup>C for one hour. After that interval, a one ml aliquot of the medium was removed and stored frozen at -80<sup>0</sup>C until all samples were collected. The medium was replaced on each plate so the final volume was 4 ml until the next time point. The mTNF that was secreted into the medium was assayed using a commercial ELISA assay kit obtained from Biosource, #KMC3012. The assay was conducted according to the manufacturer's instructions. Each sample was determined in duplicate and each time point was collected from 4 different plates of infected cells.

Western Blot analysis: For western blot analysis. A549 cells on a 6 cm plate were infected at an M.O.I. of 10. At various times post-infection, the cells were scraped and collected as described above. The cells pellet was stored at -80<sup>0</sup>C. Three hundred ul of lysis buffer was added to each sample, freeze/thawed 3 times. and passed through a 22-gauge needle. A Bradford assay was performed to determine the quantity of protein. Ten micrograms of total protein was loaded onto a 4-20% SDS/PAGE gel and electrophoresed. The proteins were transferred onto a PVDF membrane. blocked with 3% dry milk in PBS, and blotted with the appropriate antibody. The antibodies for the E3 proteins and for pVIII (a protein made during the late times of infection) were polyclonal rabbit antibodies. These were used at 1:400. The antibody for murine TNF was obtained from "R and D Systems" and was used at 0.1 ug/ml. The appropriate secondary antibodies were used and then visualized using the ECL system (Amersham).

In addition to Western analysis of cell lysates, the medium that was collected at hourly intervals was analyzed by western blots using the same anti-mTNF antibody and 25 ul of medium loaded per lane.

**Table 4**

Oligonucleotides used to amplify the CD and mTNF genes ( sequences are 5' to 3').

CD2-NdeC	GCTGCAAGTGCTGCACATGGGCTGCATG
PacCD	GTGAGCTTAATTAAAGGCTAGCAATGTCGAATAACGC
PmeCD	GTGAGCGTTAACACAGTCGTTAACGTTGTAATCG
NheCD	GGCCGCTAGCGGCTAACAAATGTCGAATAACGC
SunCD	GTGAGCCGTACGAGGCTAGCAATGTCGAATAACGC
MunCD	GTGAGCCAATTGCAGTCGTTAACGTTGTAATCG
BstBICD	GCGCTTCGAAGTGGAGGCTAACAAATGTCGAATA
StuICD	GGCCAGGCCCTAAAGCTCGCTGTAACCCAGTCG
5BamTNF	GGCCGGATCCGACACCATGAGCACAGAAAGCATG
5ClATNF	GGCCATCGATGACACCATGAGCACAGAAAGCATG
3SwspTNF	CGCGAATATTAAATCCATTCCCTCACAGAGCAATGAC
5NheMIP3	GCGCGCTAGCCCACCATGTGCTGTACCAAGAGTTGCT
3MunMIP3	GGCCAATTGTTACATGTTCTGACTTTTACTGAG
5NheMCP3	GCGCGCTAGCCCACCATGTGGAAGCCCATGCCCTCACC
3MunMCP3	GGCCAATTGTCAAAGCTTGGAGTTGGTTTTCTTG

**Example 7**

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#### Viral Expression of CD or mTNF

CD-containing viruses: E3-CD-PacPme (Onyx 301), E3-CD-NhePme (Onyx 302), E3-CD-SunMun (Onyx 303), E3-CD-NheMun (Onyx 304). The cell line A549 was infected with each of the viruses Onyx 301, Onyx 302, Onyx 303, and Onyx 304 at a M.O.I.(multiplicity of infection) of ten. At the designated hours post infection (p.i.), samples were harvested as described in methods section for assay of CD activity. Also at each time point, a picture was taken of the cells to show phenotypic differences.

10 Figure 8 shows control mock infected and Ad5-infected cells, and figure 9 shows cells infected with viruses with CD inserted into the gp19K region, that is, viruses , Onyx 301, Onyx 302, Onyx 303, and Onyx 304. The wild type infection proceeds normally and shows almost total CPE by 48 hr p.i. The Onyx 304 virus shows near wild type levels of CPE at 48 hr p.i.. while Onyx 303 shows a slightly

attenuated infection. The other two viruses, Onyx 301 and Onyx 302, show an intermediate phenotype. Interestingly, Onyx 303 is the CD substitution using the SunI site and the infection is slower than wild type likely due to the deletion of part of the y-leader, abrogating its addition to late messages and probably lowering their efficiency of translation. The viruses Onyx 301 and Onyx 302 lag behind Ad5 5 only slightly. It is noteworthy that 22 base pairs of the y-leader is deleted in the case of Onyx 301, whereas Onyx 302 contains the entire y-leader.

Experiments show that the time of expression of heterologous genes inserted in the E3 viral constructs of the instant invention is similar to the endogenous viral genes that they replace. The viruses 10 301, 302, 303, and 304 are substitutions of gp19K. As shown in fig 10, gp19K synthesis begins between 4 and 8 hours post-infection, as detected by Western blot analysis. This is similar to published values. Figure 10 also shows that the CD viruses do not make gp19K, as predicted, since the gene has been deleted.

To check when CD was first expressed, 5 ug of total protein were used per reaction. The results are shown in fig 11. As with gp19K, the CD activity is seen as early as 8 hr p.i. in viruses 301 through 15 304. This validates that endogenous expression time is similar to the inserted CD gene.

To get an idea of the amount of CD protein being synthesized from each position, 0.6 ug were used for each reaction and the results are shown in fig 12. In order to be able to compare between the different viruses, a smaller amount of protein was used in to ensure that substrate conversion was incomplete. Fig 12 shows that Onyx 301, Onyx 302, and Onyx 304 synthesize similar amounts of CD. 20 On the other hand, Onyx 303 shows total conversion of substrate at about 36 hours, indicating that there is proportionally more CD synthesized by Onyx 303 infected cells compared to the others.

To ensure that the remaining E3 genes still express their corresponding proteins, A549 cells were infected at an MOI of 10 with Ad5, Onyx 303 or Onyx 304, and the cells were harvested at various times post-infection. The proteins were extracted from the cell pellets, run on a gel, transferred, and 25 analyzed by Western blot using the appropriate antibody. The results are shown in figure 16 and figure 19, for Ad 5, and Onyx 303 and Onyx 304, respectively. Onyx 304 produces wild type levels of the E3 proteins, namely 11.6K, 14.5K, and 14.7K. Interestingly, Onyx 303 produces almost no 11.6K, which could account for its attenuated phenotype. Without wishing to be held to any particular theory, we speculate that because the y-leader is almost completely missing as a result of the insertion of CD, that 30 the message for 11.6K is not translated efficiently. Although Onyx 303 appears to synthesize Ad5 wild type levels of 14.7K, both the level and processing of the 14.5K protein are altered.

ADP substitutions: CD and mTNF: The E3-Adenovirus Death Protein (ADP) gene was replaced with either CD or mTNF, as described above. It is known that ADP deleted viruses do not lyse infected cells at the expected time compared to wild type. Thus, the gene is thought to be important in virus release. As with the ADP deletion viruses, the invention viruses which replace other genes in this region show a similar phenotype. This is shown in figure 13 and figure 14. At 72 hr p.i. when the wild type infection shows total CPE, virus Onyx 305 (CD insertion) and virus Onyx 320 (mTNF insertion) exhibit significant but reduced CPE compared to Ad5. The infection doesn't reach total CPE until 96 hr p.i. (figure 14). If the medium is changed every 24 hrs, the cells remain attached and exhibit an almost normal phenotype even at 120 hr p.i.. It is not until after 164 hr p.i. (7 days) that the cells appear to show classic CPE and come off the dish. This is a key observation and will be useful in a therapeutic sense since even if infected cells do not lyse immediately, they will nevertheless continue to express the heterologous gene of interest.

ADP is predominately a late protein whose expression is thought to be driven by the major late promoter and expressed at high levels during the late phase, that is, after DNA replication. To determine if inserted foreign genes show similar kinetics, a CD assay was performed on Onyx 305, and analysis for mTNF was performed on Onyx 320. As shown in figure 11, Onyx 305 does not show substantial CD activity until 12 hr p.i., a time when the virus has entered into the late phase. Also contrasted in figure 11 is the comparison with CD in the gp19K region, an early region. Clearly, there is a difference in the timing that the protein is synthesized.

To compare the amount of CD activity, a CD assay was performed using 0.6 ug of total protein in each reaction (figure 15). Upon visual inspection and comparison to figure 12 (experiments were done at the same time), it appears that Onyx 305 does not synthesize quite as much as Onyx 301-304. But it should be noted that the Onyx 305-infected cells have an attenuated course of infection, and therefore, importantly, will synthesize CD for a considerably longer period of time.

As a check for late stage of infection, Western blot analysis was performed on cell lysates and blotted with an antibody to ADP (11.6K), as well as to a late structural protein, pVIII. Both 11.6K (figure 16) and pVIII (figure 18) expression was seen in Ad5 infected cells. pVIII expression was also observed in Onyx 320 infections occurring at 24 hr p.i. (See, figure 18), indicating that the virus had entered into the late phase between 12 and 24 hr p.i. Also note that Onyx 305 fails to produce ADP, as expected, since it does not contain the gene (See, figure 20).

CD inserted in place of ADP exhibits a similar time to expression as ADP. A similar observation was made with Onyx 320, which has mTNF inserted in place of ADP. Briefly, cells were infected and lysates made at different times p.i. Western blot analysis was performed on these lysates, using both mock-infected and Ad5-infected cells as controls. It was shown that intracellular mTNF expression was

not seen until after 24 hr p.i. See, Figure 21). This is consistent with our previous findings that generally foreign genes mirror the expression profiles of the endogenous adenoviral gene that they replace.

#### Example 8

##### Expression of mTNF in Onyx 320 Mirrors Endogenous Late Gene Expression

5 Additional experiments were conducted to quantify the expression of mTNF from Onyx 320, and to confirm that mTNF exhibits a late expression pattern similar to 11.6K.

mTNF Quantification: A549 cells were infected with Onyx 320 at an m.o.i. of 10 and the cells were harvested at the indicated times post-infection. To measure secretion of mTNF, the medium was removed one hour prior to the indicated time point and replaced with 5 ml of fresh medium. Aliquots of 10 the newly added medium were then removed at the end of that hour to obtain data indicating the amount of mTNF produced per hour. As a control, Ad5 and mock infections were also conducted. Figure 20 shows Western blot analysis of the timing and relative levels of expression of the endogenous E3 gene, 11.6K. Based on the detection limits of this assay, and previous published data, 11.6K is predominately synthesized after the start of the late phase of infection. Figure 21 shows Western blot analysis of 15 intracellular mTNF. This is the 27 kD uncleaved form of the molecule. See, Kriegler et al., Cell, 1988 Apr 8;53(1):45-53. As is true of the 11.6K protein, mTNF expressed from this region is predominately synthesized and detected during the late phase of infection.

Figure 22 shows Western blot analysis of mTNF secreted into the medium during the one hour preceding the indicated time points. Twenty-five ul of culture medium was run on the gel to obtain this 20 result. This shows that again detectable levels of the mTNF are produced at late times post-infection. Further, the mTNF produced is properly cleaved as it runs at the predicted molecular weight of 17kD. This blot also shows that *de novo* synthesis was still occurring up to 144 hr p. i.

To quantitate the amount of mTNF secreted, aliquots were collected during the one hour time points and the mTNF present was quantitated using a commercially available ELISA kit. Figure 23 25 shows the results of 4 different experiments, and the results are expressed as the amount of mTNF produced per hour per one million cells. As shown in the figure the infected cells express high levels of mTNF: indeed, about 43 to 68 nanograms were measured during the highest production period.

To address the issue of expression of the remaining E3 proteins, Western blot analysis was performed to detect gp19K, 14.5K, and 14.7K from Onyx 320, and Ad5-infected cells. As shown in 30 figure 24, Onyx 320 synthesizes similar levels to Ad5 of the E3 genes, gp 19K, 14.5K, and 14.7K; however, the processing of 14.5K is altered.

mTNF is Expressed as a Late Protein: An important characteristic of adenoviral late protein expression is that it is dependent on viral DNA synthesis. Thus, a classic experiment to show *bona fide* late protein expression is to determine whether or not expression occurs in the presence of araC, an 35 inhibitor of DNA replication. Figure 17 shows the expression of gp19K, 11.6K, 14.5K and 14.7K in the

presence and absence of araC in cells infected with Ad5. Note that there is little or no detectable expression of the late protein 11.6K. Thus, to determine if mTNF when inserted in place of the 11.6K exhibited a late protein expression pattern, experiments were conducted to measure expression in the presence of araC. Figure 25 shows that intracellular mTNF is not expressed in the presence of araC, whereas it is in its absence, indicating that mTNF is indeed displaying a late protein expression pattern. As a control, figure 27 shows that an early protein, gp19K, is synthesized in the presence of araC by both Ad5 (See, also figure 17), and Onyx-320. Lastly, another control was run to further confirm that Onyx 320 infection truly reaches late phase in the absence of araC. Western blot analysis was performed against another known late protein, pVIII. Figure 26 shows that pVIII is not expressed in the presence of araC, but is in its absence. This further supports that mTNF is expressed as a true late protein. Note that figure 26 also shows the expression pattern for pVIII in Onyx 304 and Onyx 305, and the results with these viruses are similar to those observed for Onyx 320.

#### Example 9

##### Expression of mTNF from the E3B Region of Onyx 321

As described above, Onyx 321 has the entire E3B region substituted with mTNF using the engineered sites Clal and Swal. Experiments were conducted to determine the expression properties of mTNF from this region of the virus. A549 cells were infected at an MOI of 10 and photographed at the indicated times post-infection (Figure 28). At the 48 hour time point, Onyx-321 showed greater CPE than wild type virus at the same time post-infection.

Next, mTNF expression was analyzed along with the remaining E3 proteins. These blots are shown in figures 29 and 30. In figure 29, mTNF expression was not detected until 24 hr p. i., unlike 2 of the genes it replaced, namely 14.7K and 14.5K, which appear about 8 to 12 hr p. i. In this case, detectable mTNF expression was later than endogenous gene expression since 3 genes were removed, along with endogenous splicing signals. Figure 30 shows that while gp19K expression remains similar to Ad5, the expression of 11.6K by Onyx 321 is greatly enhanced. Without wishing to be helpful to any particular theory, it can be speculated that this is the cause for the appearance of the enhanced CPE seen in figure 28.

Since mTNF did not appear until 24 hr p.i., the question arose if it is being expressed as a late protein? To test this, araC was added to the culture medium and cell lysates were analyzed by Western blot. The results are shown in figures 31 and 32. Murine TNF in the place of E3B appears to be a *bona fide* late protein as its expression was dependent on DNA replication. As controls, gp 19K expression continued in the presence of araC, while that of 11.6K did not. This represents another region for insertions of transgenes that we wish to express predominately during the late phase of infection.

Example 10Creation of Viruses Containing Genes for Chemokines

The genes for the chemokines hMCP3-alpha and hMIP3 alpha were inserted into the 6.7K/gp19K region and viruses were made. Specifically, the oligonucleotides used to PCR amplify these genes are listed in Table 4; each of these genes were amplified with the NheI site on the 5' end and the MunI site on the 3' end. The MIP3-alpha gene was obtained in the form of an EST from ATCC (EST# 113153) and the EST for MCP3-alpha was obtained from Genome Systems (Image #485989). The plasmid for insertion was pG-E3SV; both the plasmid and the PCR products were cut with NheI and MunI. All fragments were gel purified. Then each of the chemokines was inserted into the vector individually. Viruses were created using these constructs just as described above. The viruses were plaque purified and confirmed by methods described above.

Example 11Construction of BstLink Virus/TP-DNA

Insertion of a gene of choice into the adenovirus genome is an extensive process. It involves cloning into the smaller plasmids first and then adding this into the larger, pNB-based vectors. Ideally, the smaller plasmids could be used directly. However, their use for co-transfections for virus construction is difficult because it allows a limited amount of overlapping sequences necessary for homologous recombination. For example, there is only a 240 base pair overlap in sequence between the plasmid pSN and genome viral DNA at the 5' end when cutting TP-DNA with EcoRI, the standard method. Thus, to increase the region of overlap, a virus called BstLink was created as follows. The plasmid pG-Bst→Stu was used because it deletes the 11.6K death gene, which, if used to generate viruses, results in much smaller plaques. Thus, this plasmid was digested with MunI, filled in with T4 DNA polymerase, and a BstBI linker was added by ligation. The restriction enzyme site BstBI was chosen because it is not present anywhere else in the Ad5 genome. This was built (an E3 region with no 11.6K and containing the additional BstBI site) into Ad5 (this strategy is applicable to any adenovirus, or other virus where death protein is present). TP-DNA prepared from the virus can then be used for virus construction. This is done by cutting the TP-DNA with BstBI and cotransfected with the E3 plasmid with the desired alterations. Therefore when selecting recombinant viral plaques that should contain the 11.6K gene (or another death gene), the phenotypic difference between wild type (small plaques) and the recombinant (larger plaques) will make selection of recombinants easier. In addition, this increases the homology at the 5' end to 2273 base pairs; thus overlap recombination can be used for creation of E3 virus mutants. Also recombinant viruses are easier to select based on phenotypic differences. The plaques still have to be screened for the mutations, but the proportion of correct virus clones is predicted to be higher because of the advantage of selecting for the correct construct.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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We Claim:

1. A composition of matter comprising recombinant adenoviral vectors that have restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select genes contained therein.
- 5 2. A composition of matter as described in claim 1 wherein said recombinant adenoviral vectors include restriction sites in the early region genes of the E3 region that encode the 6.7K and gp19K proteins.
3. A composition of matter as described in claim 1 wherein said recombinant adenoviral vectors include restriction sites in the early region genes of the E3 region that encode the 10.4K, 14.5K, 10 14.7K and 11.6K proteins.
4. A composition of matter comprising recombinant adenovirus, said adenovirus having restriction sites in the E3 region that facilitate partial or total deletion of the E3 region or select genes contained therein.
5. A composition of matter comprising recombinant adenovirus, said adenovirus having 15 restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select genes contained therein that encode the 6.7K and gp19K proteins.
6. A composition of matter comprising recombinant adenovirus, said adenovirus having restriction sites in the E3 region that facilitate partial or total deletion of the E3 region, or select genes contained therein that encode the 10.4K, 14.5K, 14.7K and 11.6K proteins.
- 20 7. A composition of matter comprising an adenoviral vector selected from the group consisting of pGE3SV, pGE3SV +V, pGE3SV + B, and pGE3SV +V + B.
8. A composition of matter comprising an adenovirus selected from the group consisting of E3SV, E3SV +V, E3SV + B, and E3SV +V + B.
9. A composition of matter as described in claim 1 wherein said partially or totally deleted 25 E3 region is substituted with a gene that encodes a heterologous protein, and said gene is optionally operably linked to a tissue specific promoter.
10. A composition of matter as described in claim 1 further comprising a deletion in a E1A or E1b region of said adenoviral vector, and wherein said E1b region encodes a 55k protein.
11. A composition of matter as described in claim 10 wherein said E1b or said E1A region 30 deletions are substituted with a gene that encodes a heterologous protein.
12. A composition of matter as described in claims 9 or 11 wherein said heterologous protein is selected from the group consisting of tumor necrosis factor alpha, interferon gamma, an interleukin, a cell suicide protein, and mip-3.
13. A composition of matter as described in claim 9 wherein said heterologous protein is a 35 negative selection gene.

14. A composition of matter as described in claim 13 wherein said negative selection gene is selected from the group consisting of cytosine deaminase, and thymidine kinase.

15. Cells comprising said adenoviral vectors of claim 7.

16. Cells comprising said adenoviruses of claim 8.

5 17. A method for treating cancer in a mammal in need of said treatment, comprising administering to said mammal a therapeutically effective dose of said composition of claims 9 or 11 wherein said heterologous protein has anti-cancer activity.

10 18. A method as described in claim 17 wherein said heterologous protein is selected from the group consisting of tumor necrosis factor alpha, interferon gamma, an interleukin, a cell suicide protein, and mip-3.

19. A method as described in claim 18 further comprising administering with said composition a chemotherapeutic or immunosuppressive.

15 20. A composition of matter comprising a recombinant adenoviral vector having restriction sites in the E3 region of said vector which restriction sites facilitate deletion of select genes contained in said E3 region, and substituting therefore a heterologous gene which exhibits an expression pattern similar to the deleted gene.

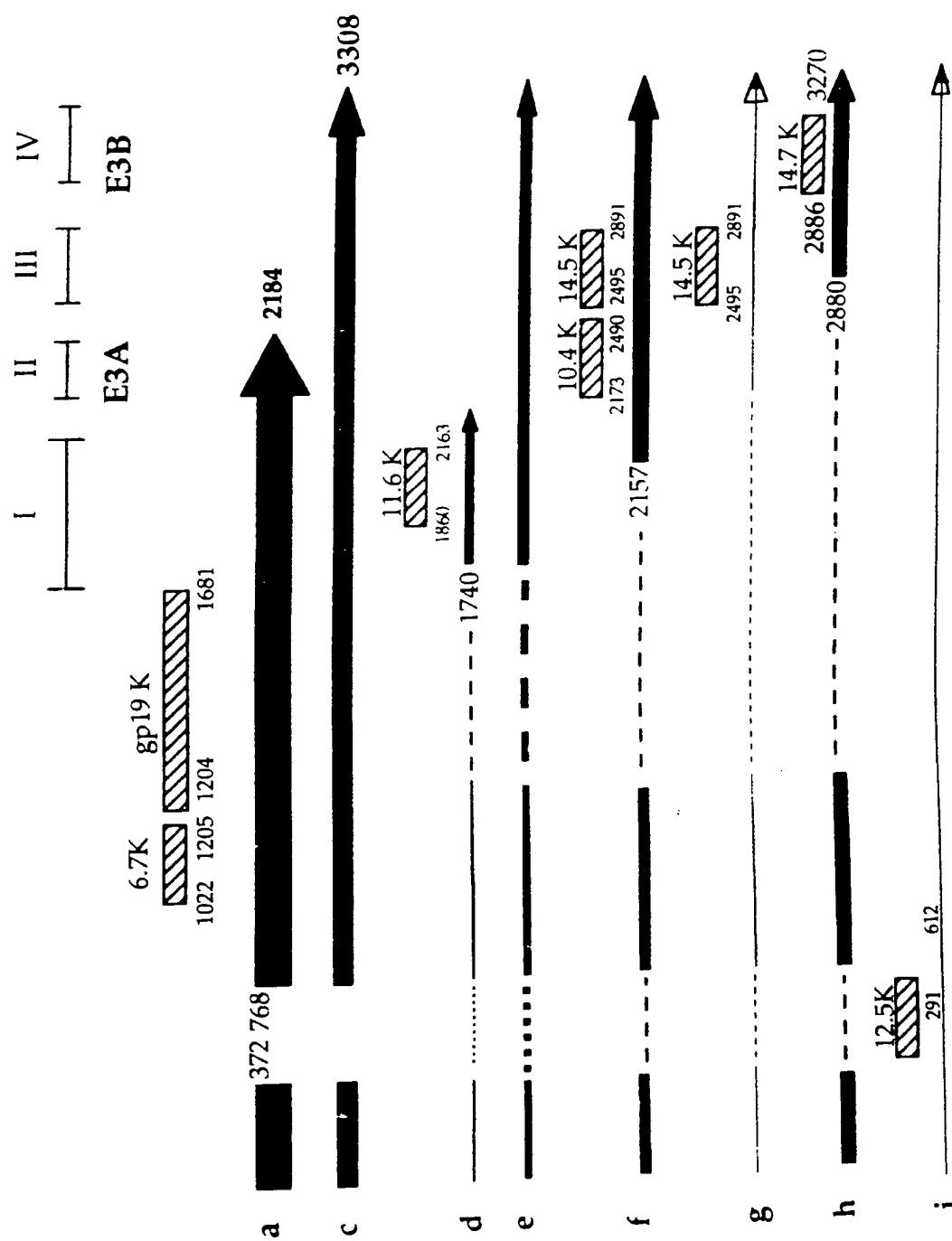


FIGURE 1

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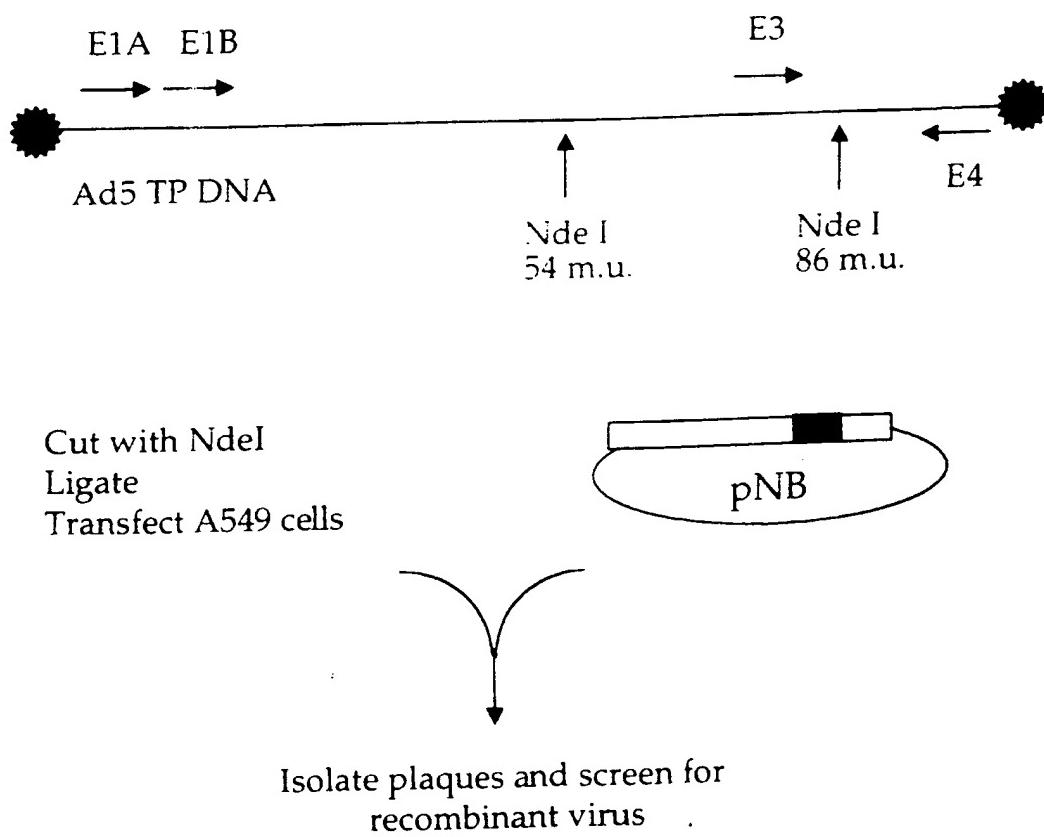


FIGURE 2

3/32

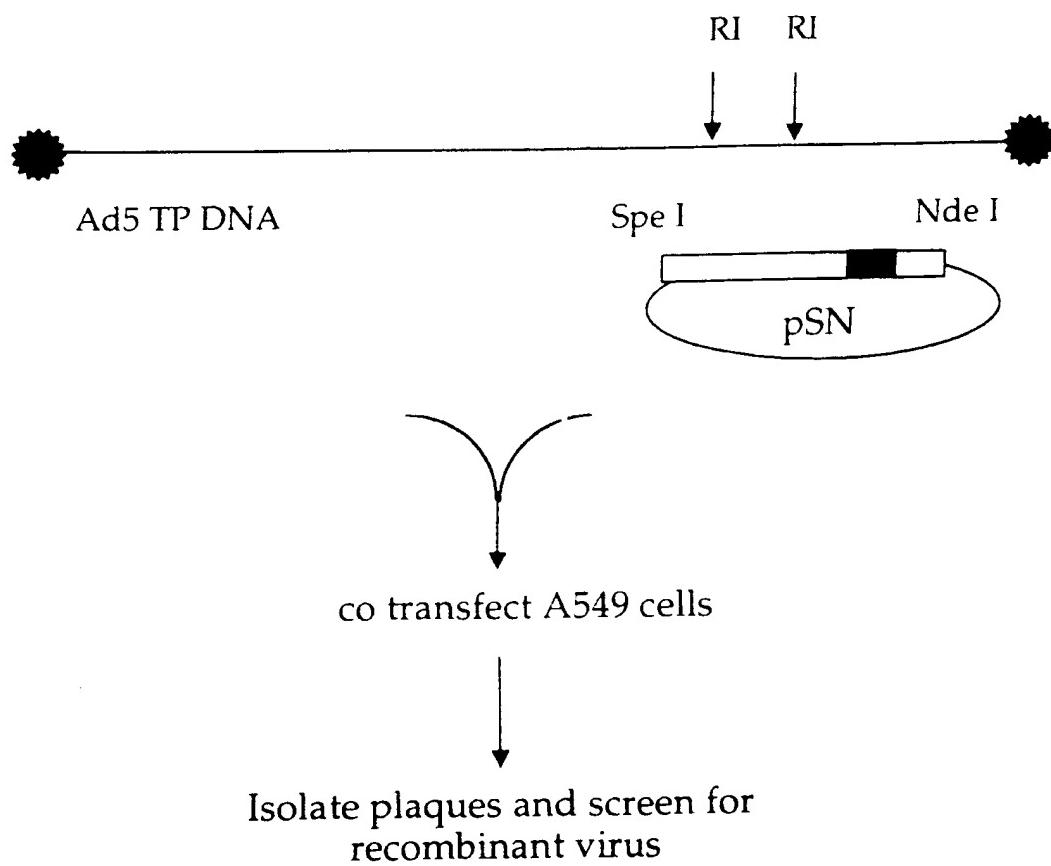
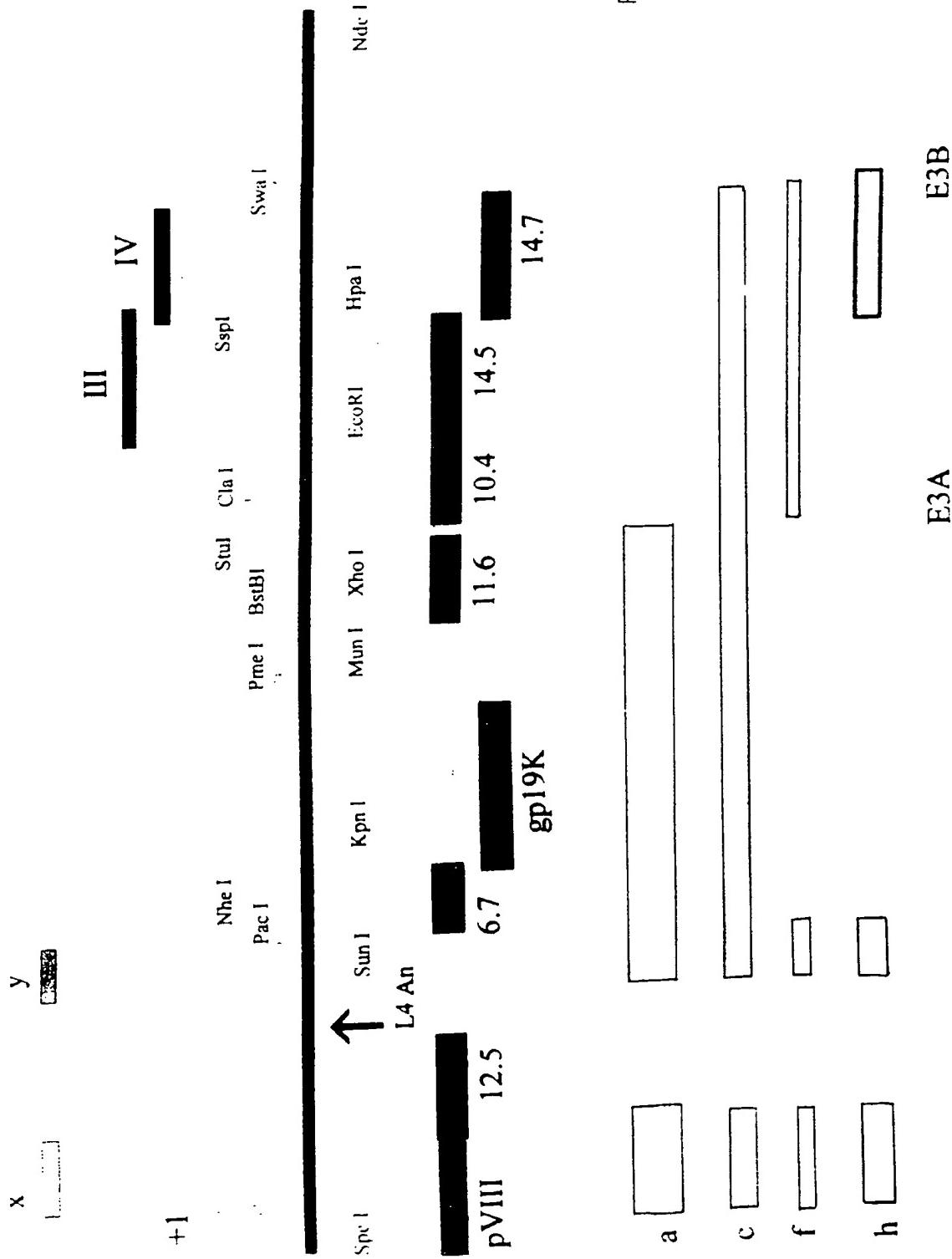
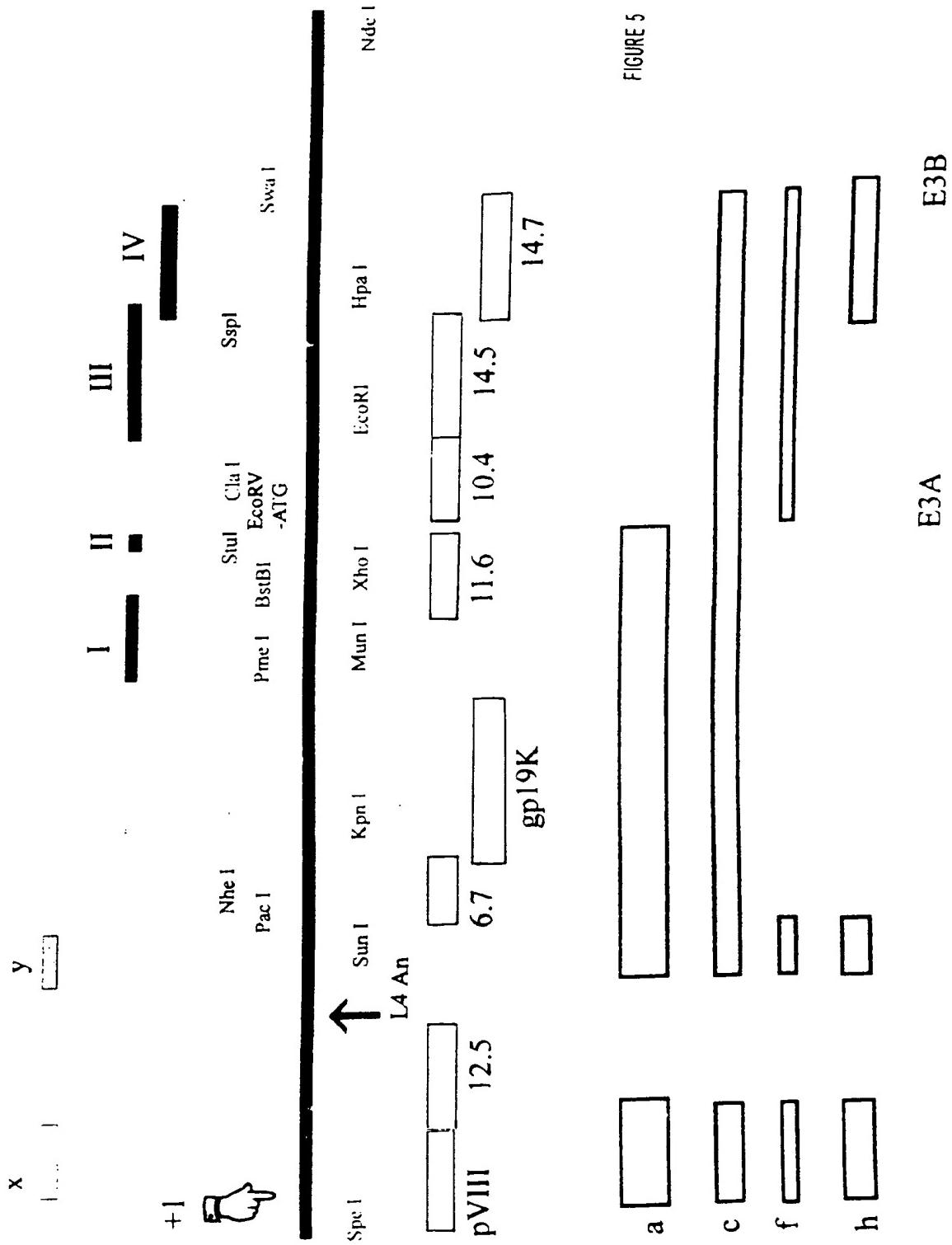


FIGURE 3

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5/32



6/32

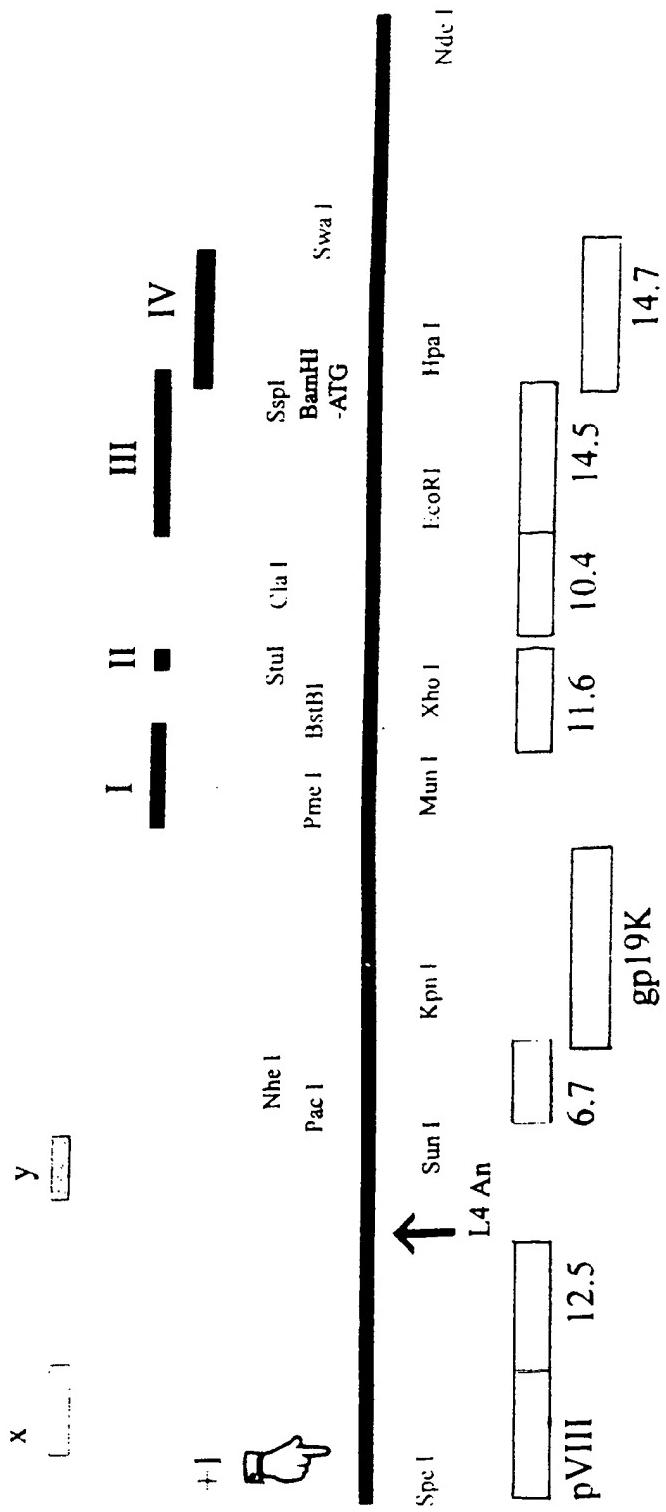
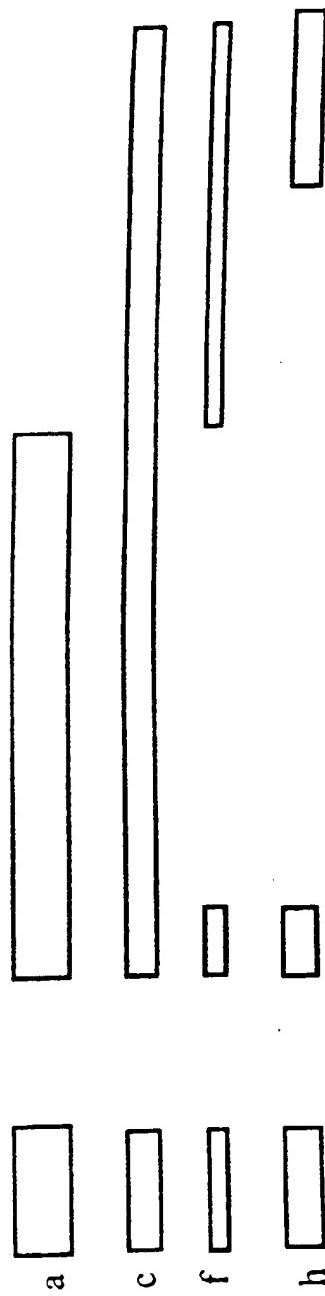


FIGURE 6



E3A

E3B

7/32

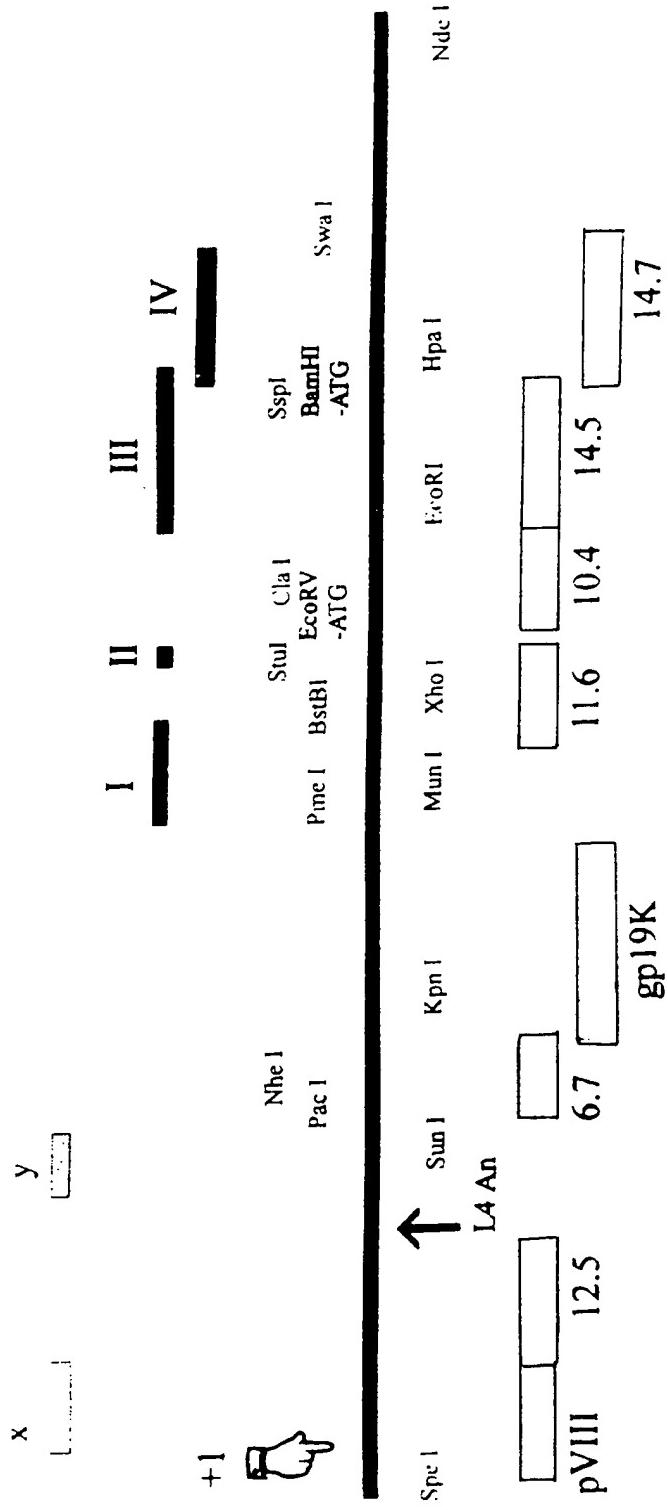
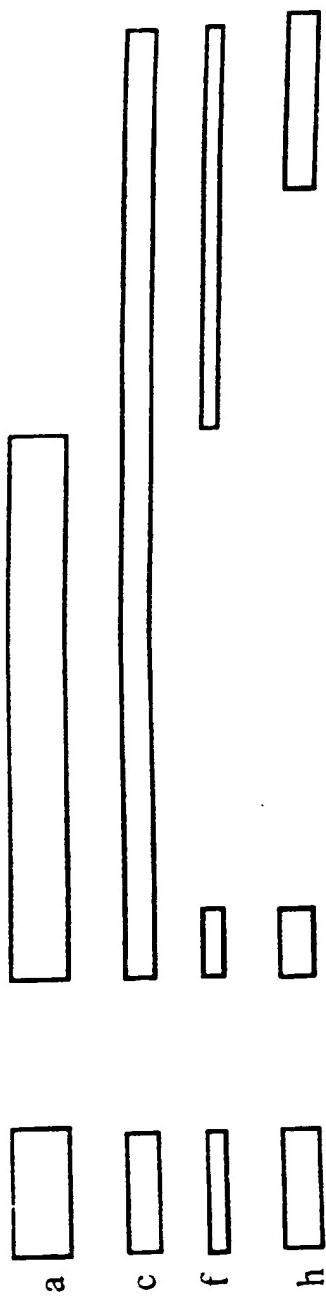


FIGURE 7

E3A  
E3B

8/32

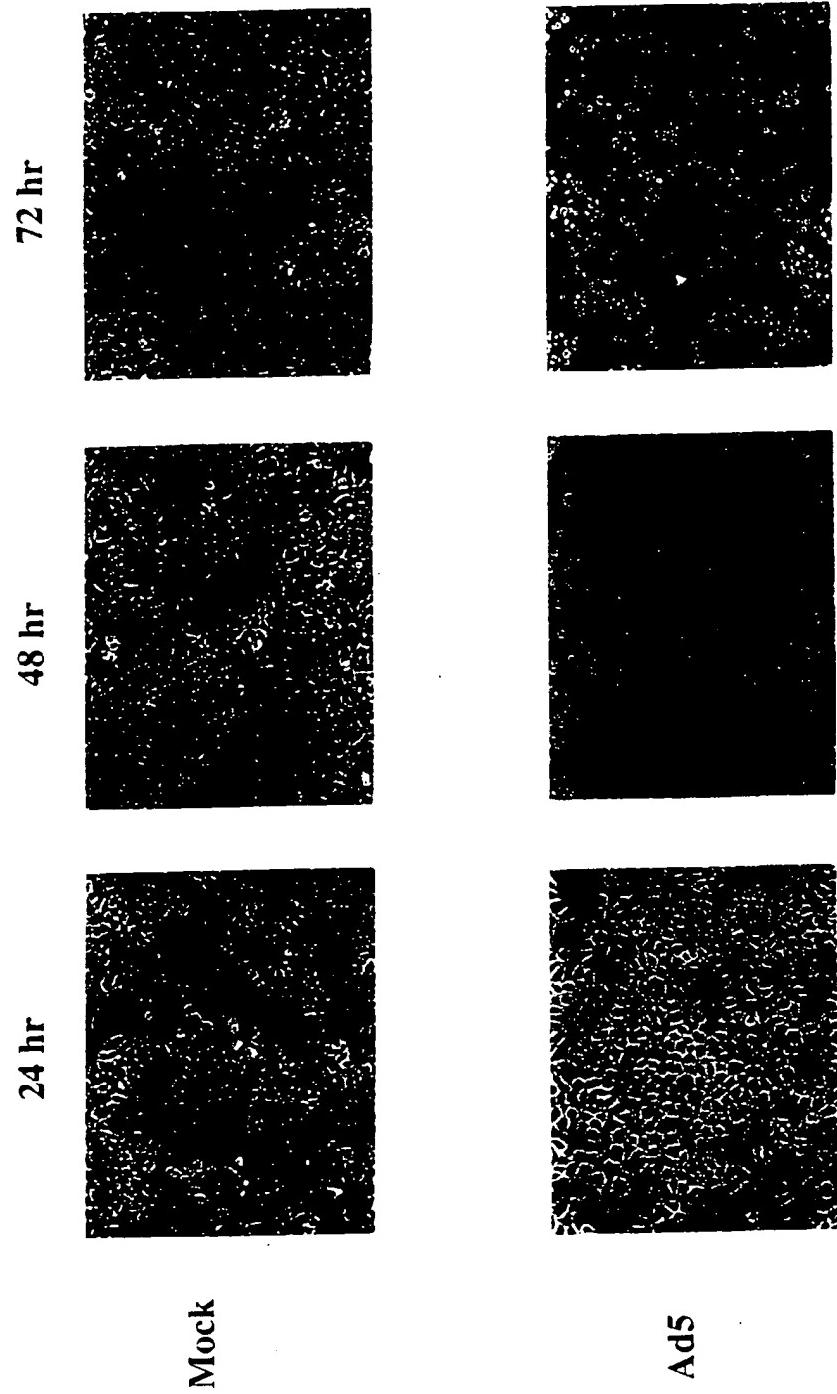
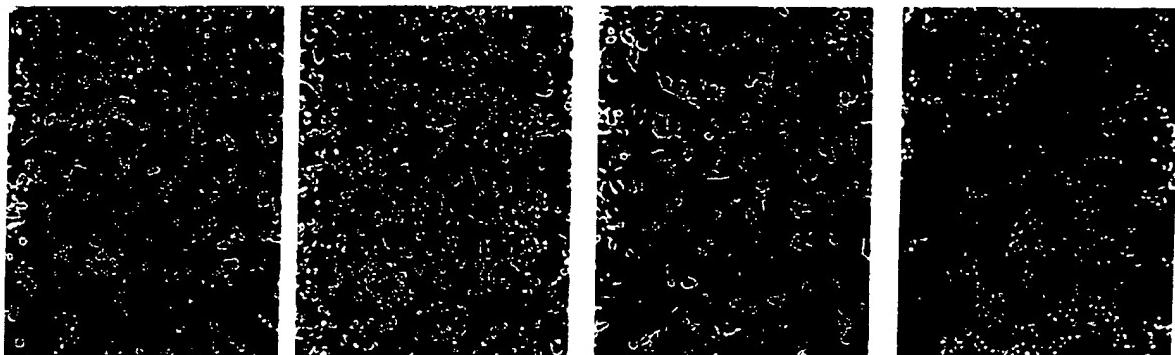


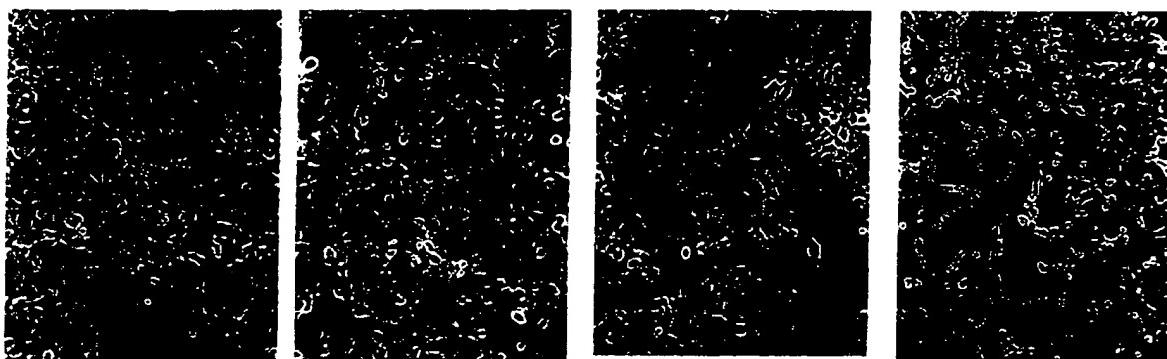
FIGURE 8

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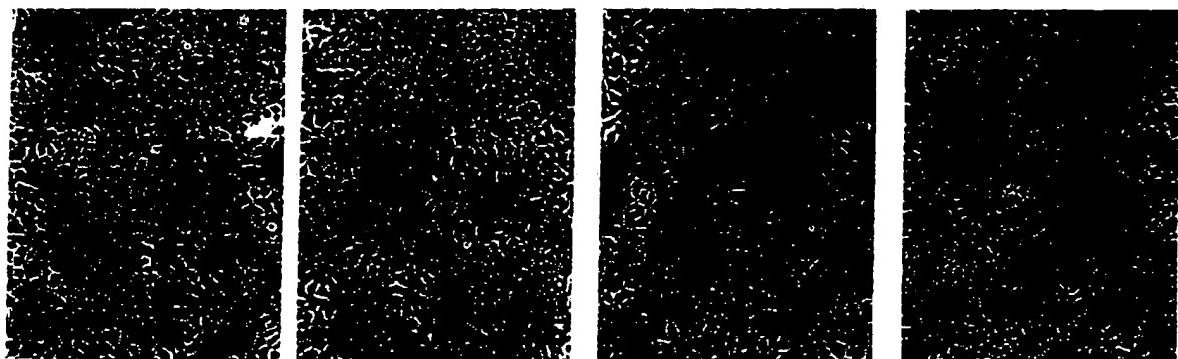
72 hr



48 hr



24 hr



301

302

303

304

FIGURE 9

10/32

## Western Blot for gp19K

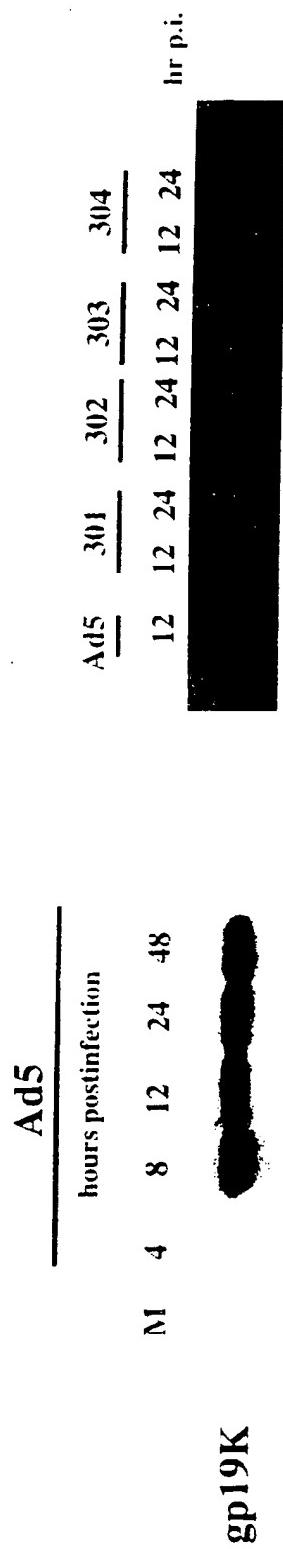


FIGURE 10

11/32

CD Assay  
*5 µg protein/rxn*

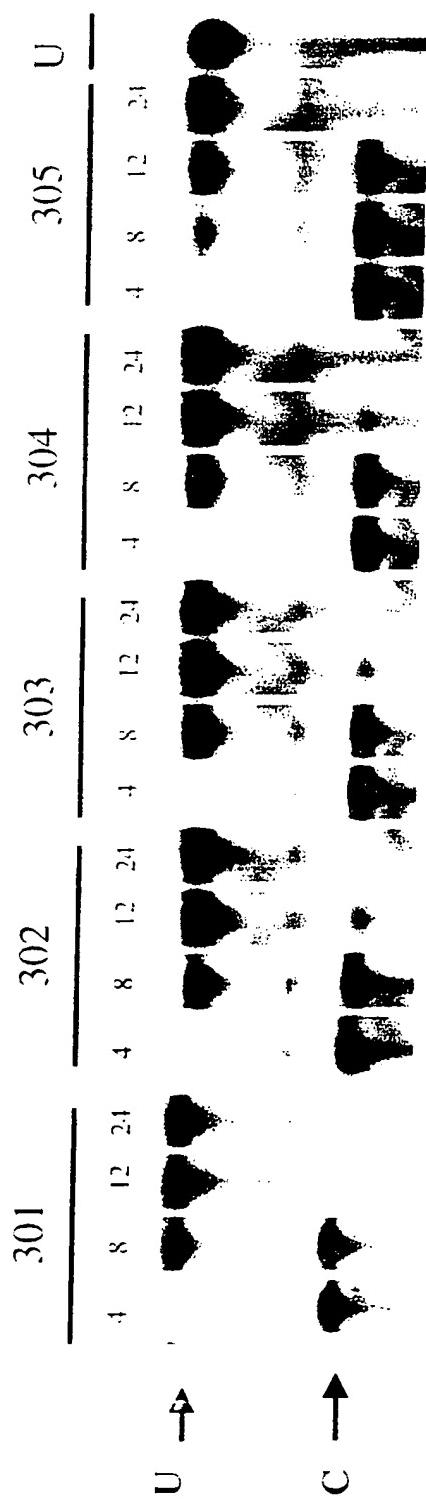


FIGURE 11

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# CD Assay

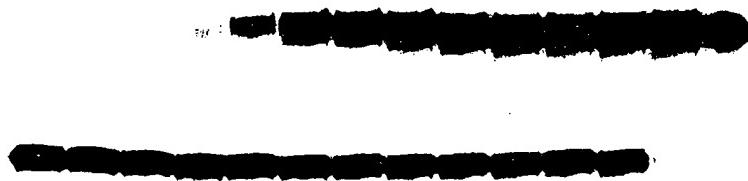
0.6 µg protein/rxn

M Ad 4 8 12 24 36 48 60 72 84 96 120 +

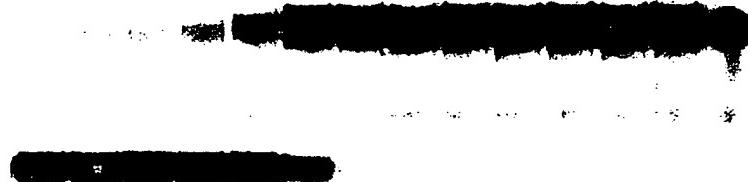
301



302



303



304

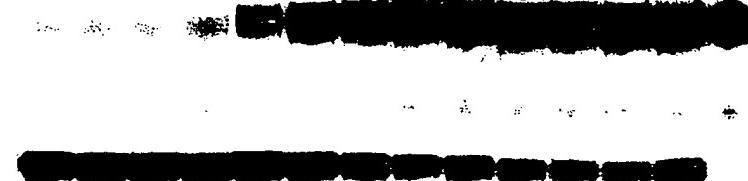
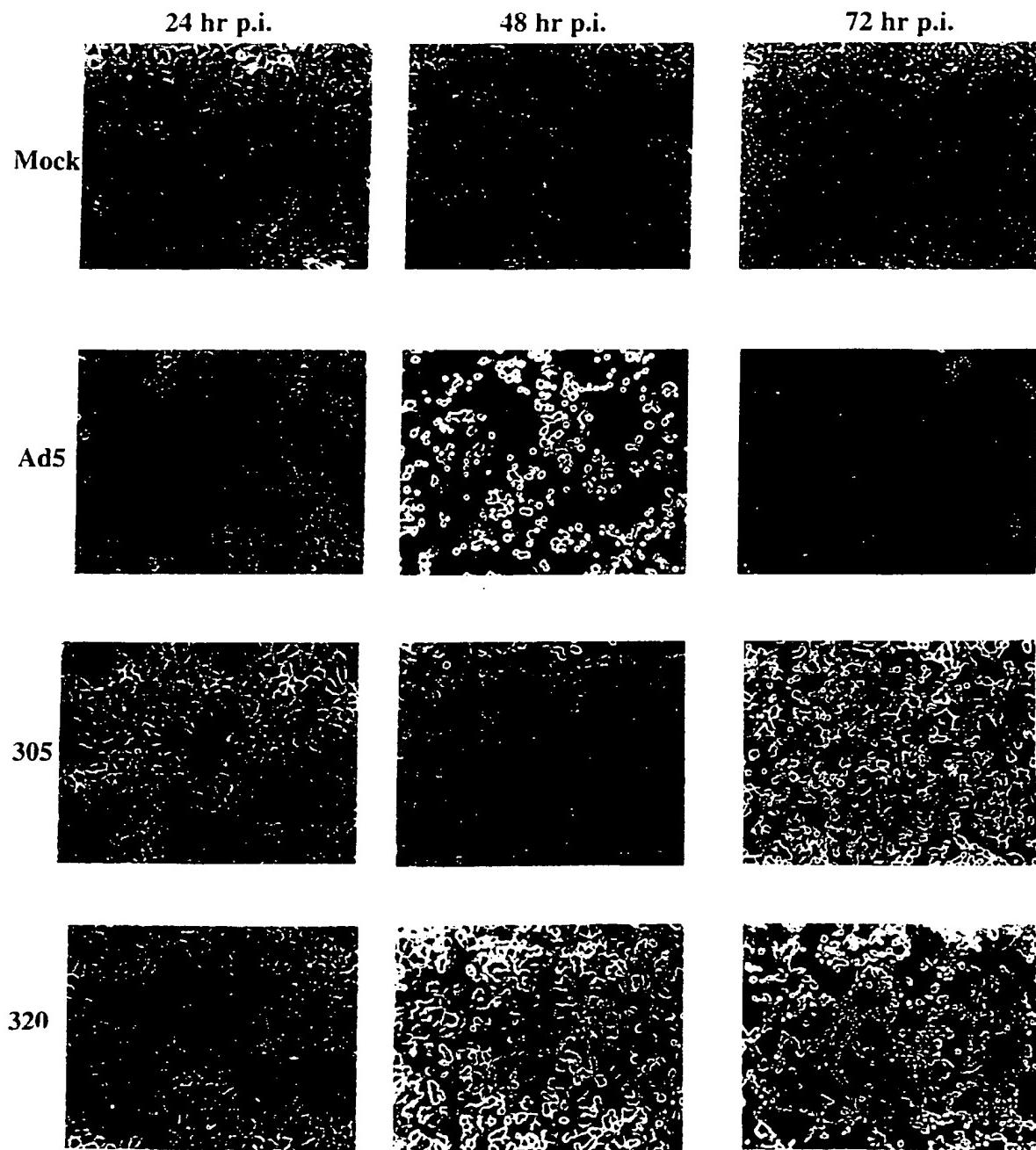
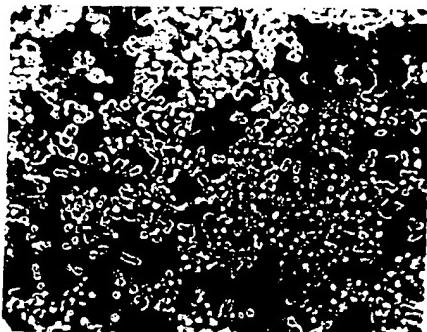


FIGURE 12

**Death Gene substitutions****FIGURE 13**

### Effect of medium change on Onyx 320-infected cells

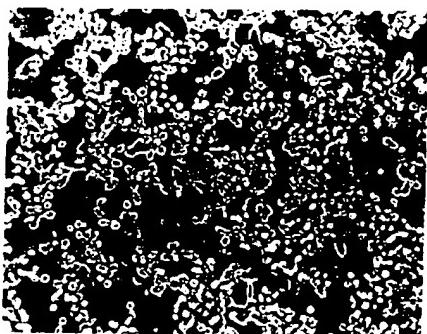
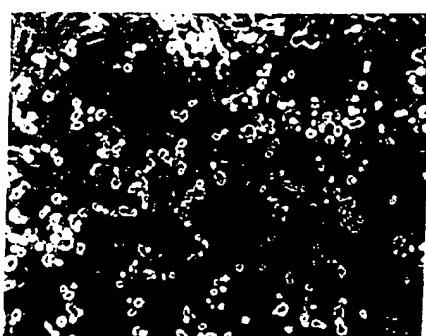
No medium change



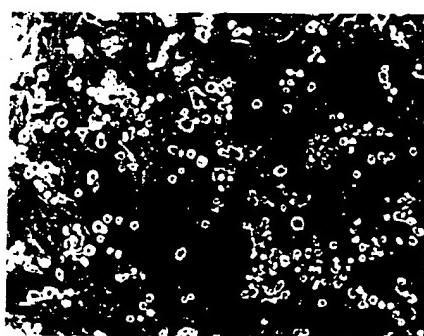
hr. p.i.

84

With medium change



96



120

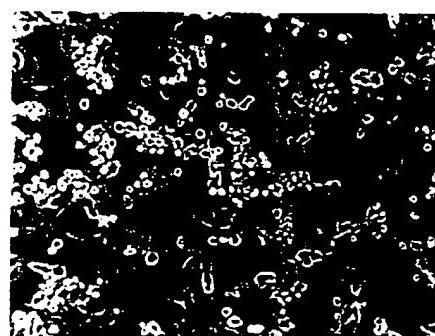
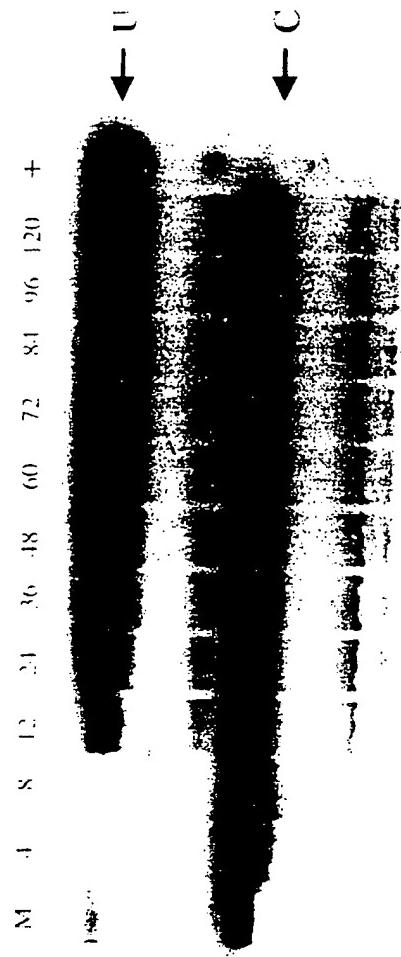


FIGURE 14

144

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## CD Assay



Onyx 305

FIGURE 15

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## Ad5 Infections

### *Expression of E3 Region Proteins*

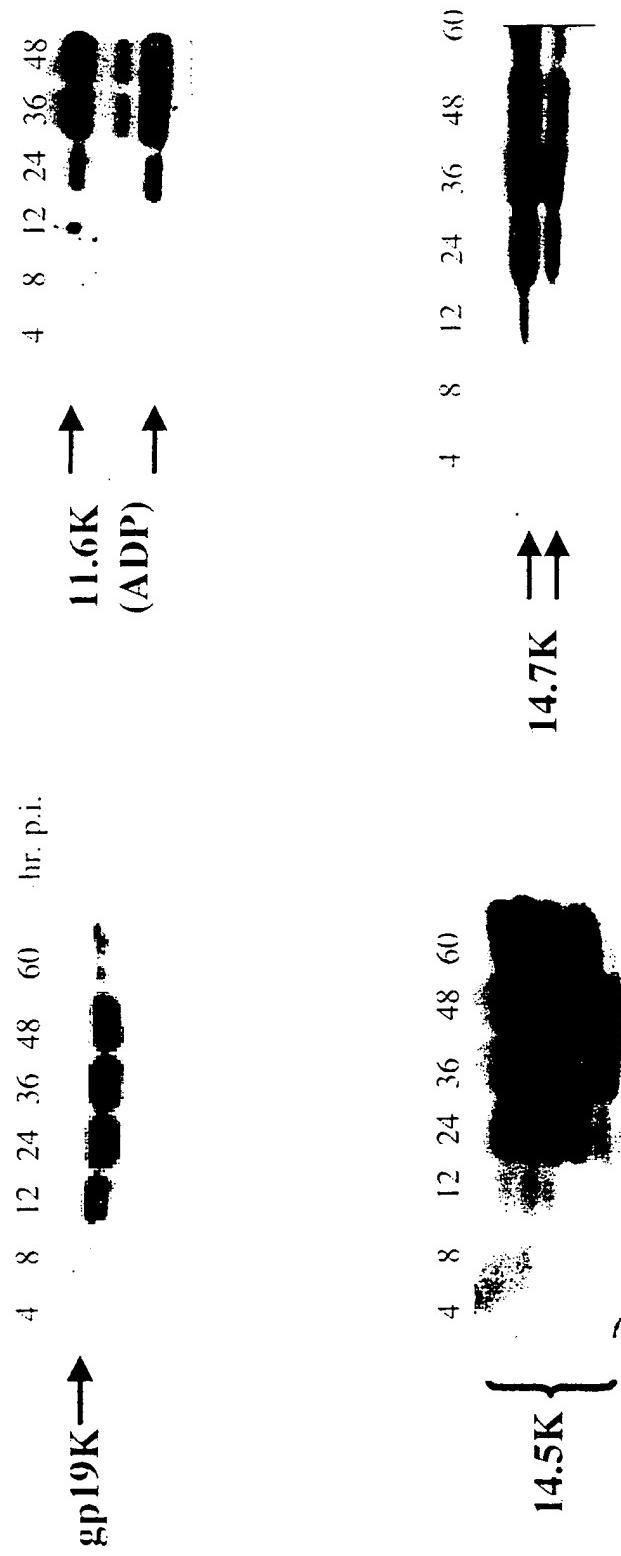


FIGURE 16

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## Infection with Ad5

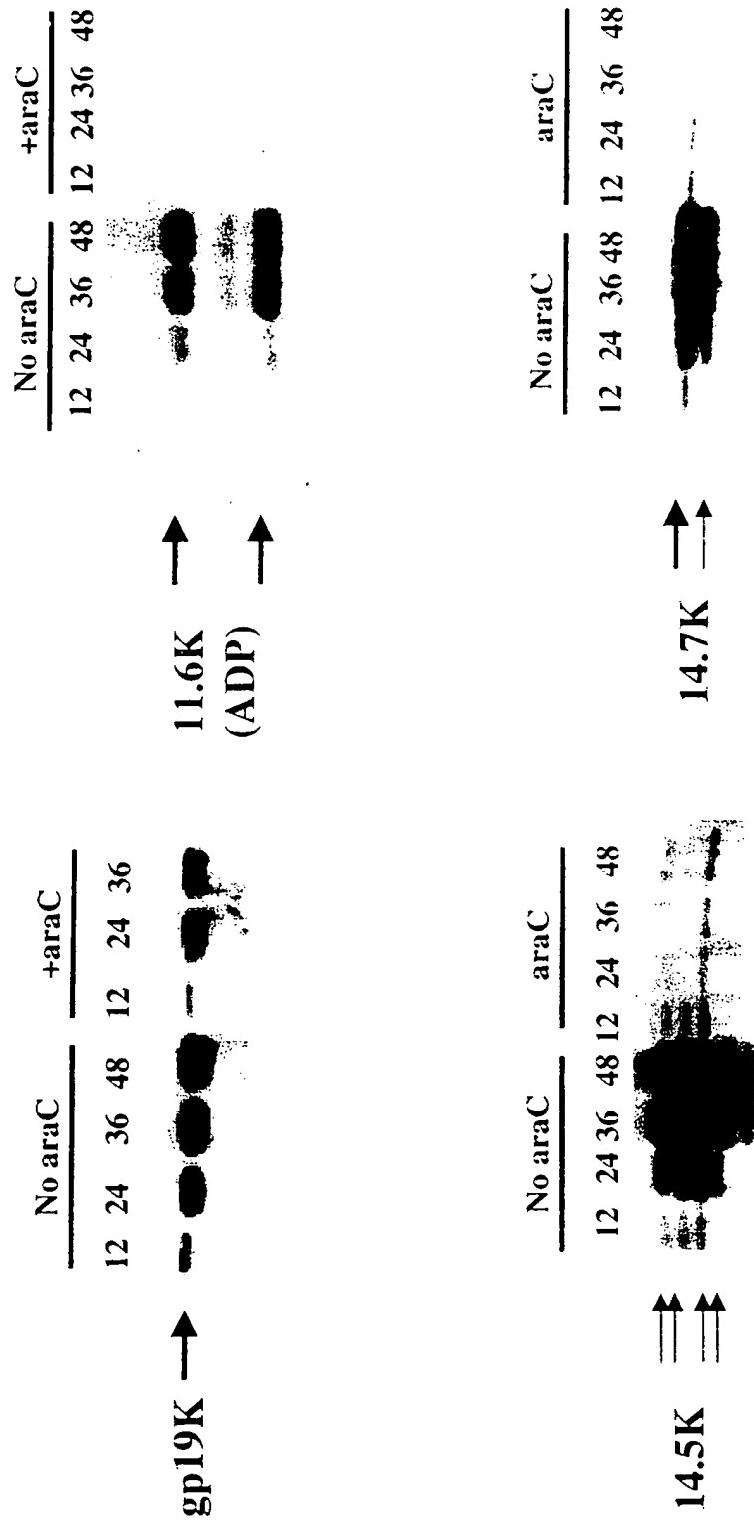


FIGURE 17

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## pVIII expression

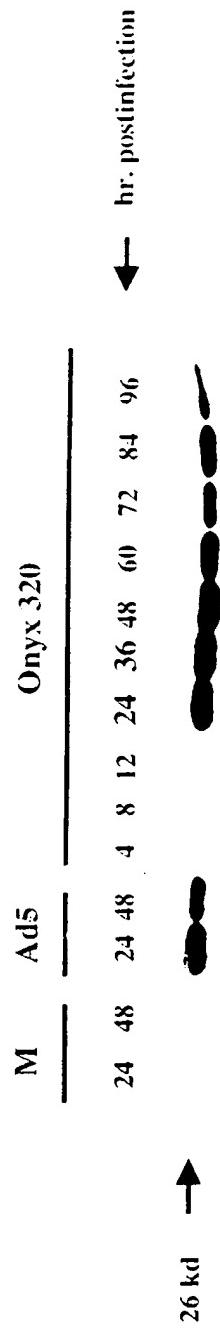


FIGURE 18

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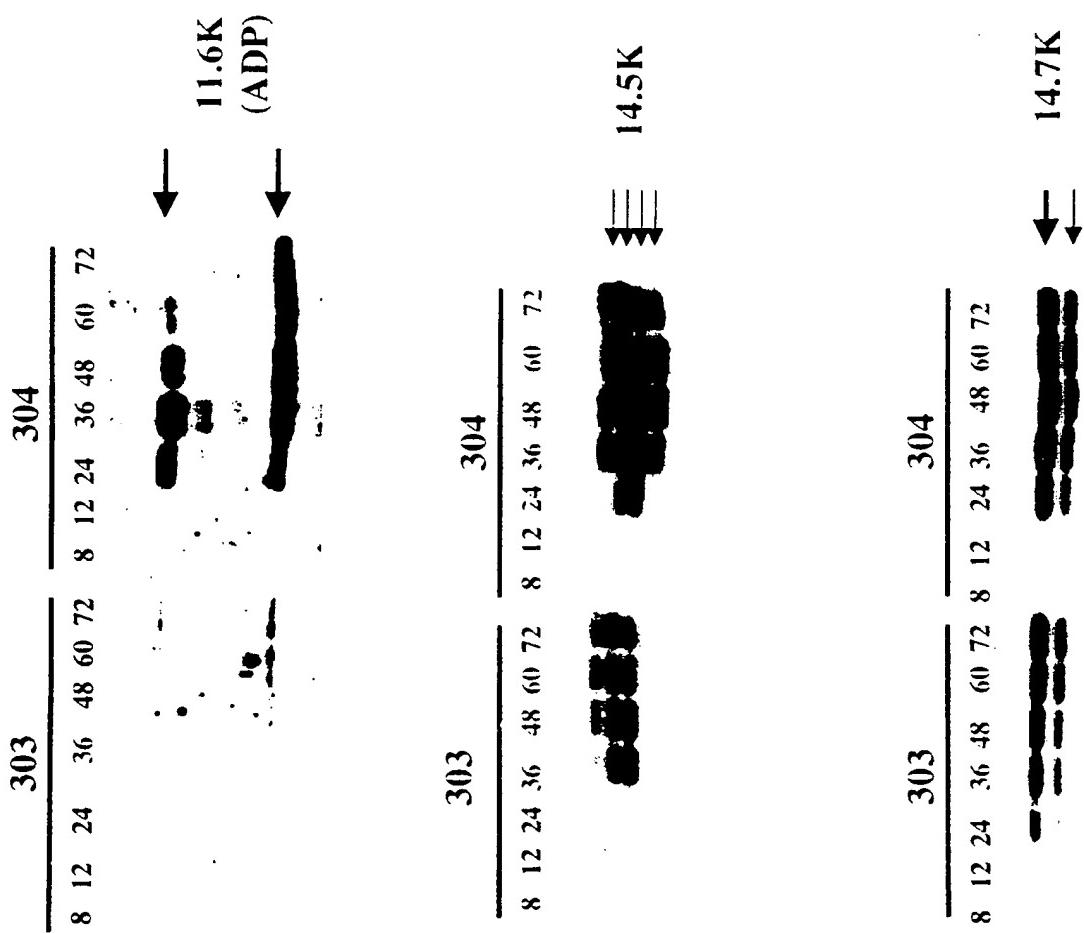


FIGURE 19

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### Western blot for 11.6K

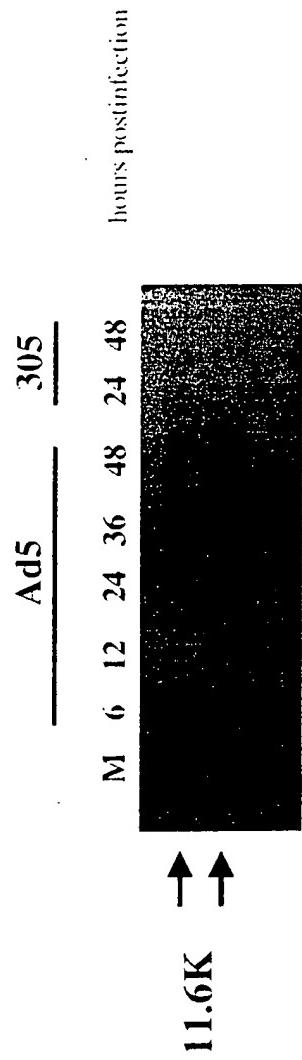


FIGURE 20

### Intracellular mTNF expression

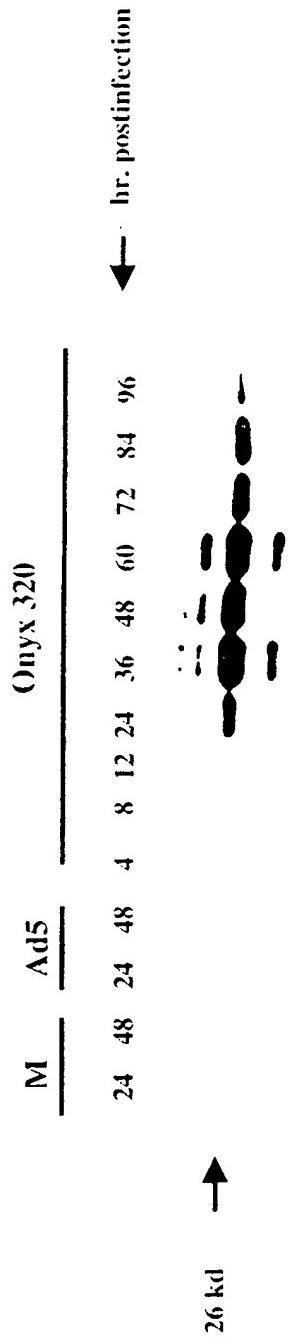


FIGURE 21

**mTNF secreted into the culture medium by ONYX 320-infected cells**

(In a one hour window)



*Method:* Medium changed one hour before indicated time. Aliquot removed after one hour for analysis.

FIGURE 22

mTNF secreted by 320-infected cells per hour

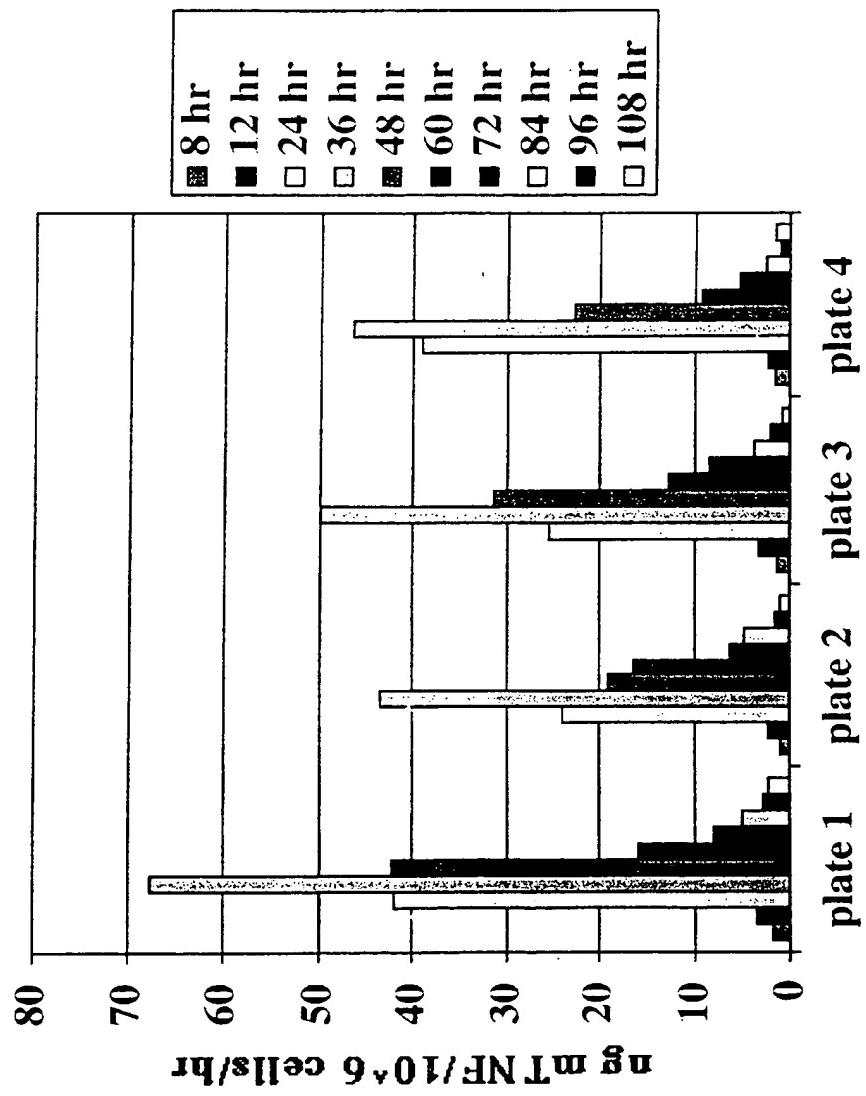


FIGURE 23

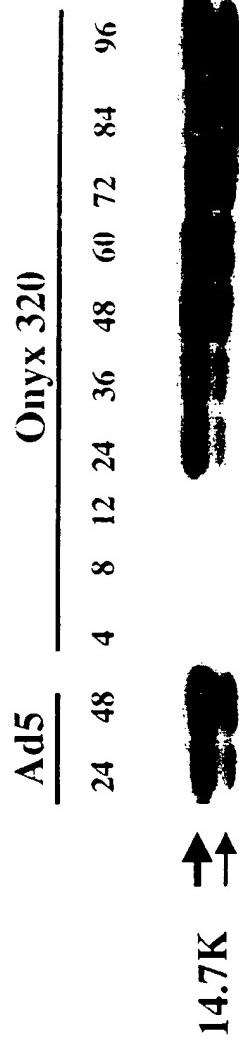
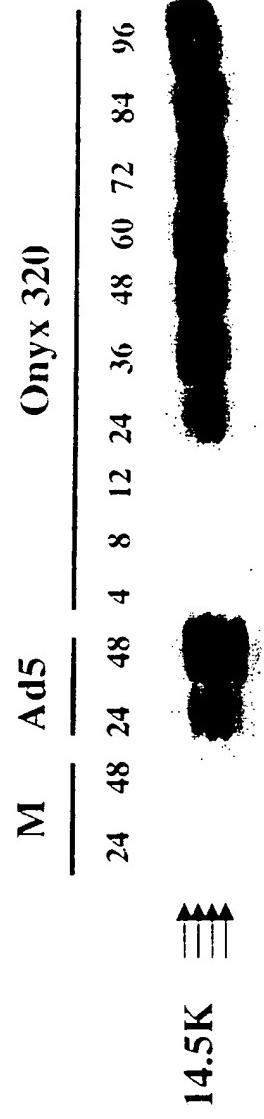
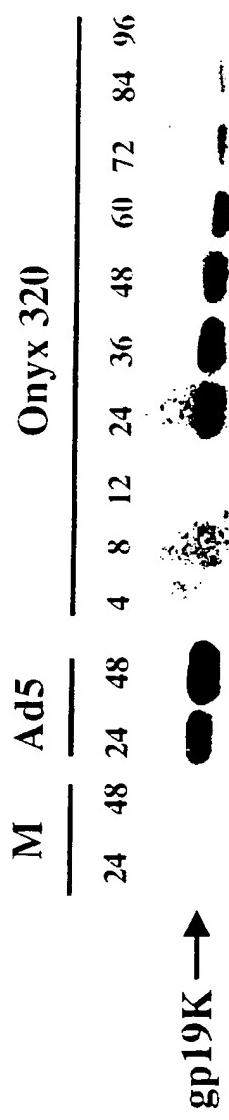


FIGURE 24

# mTNF Expression with araC

## ONYX 320



FIGURE 25

# pVIII Expression with araC

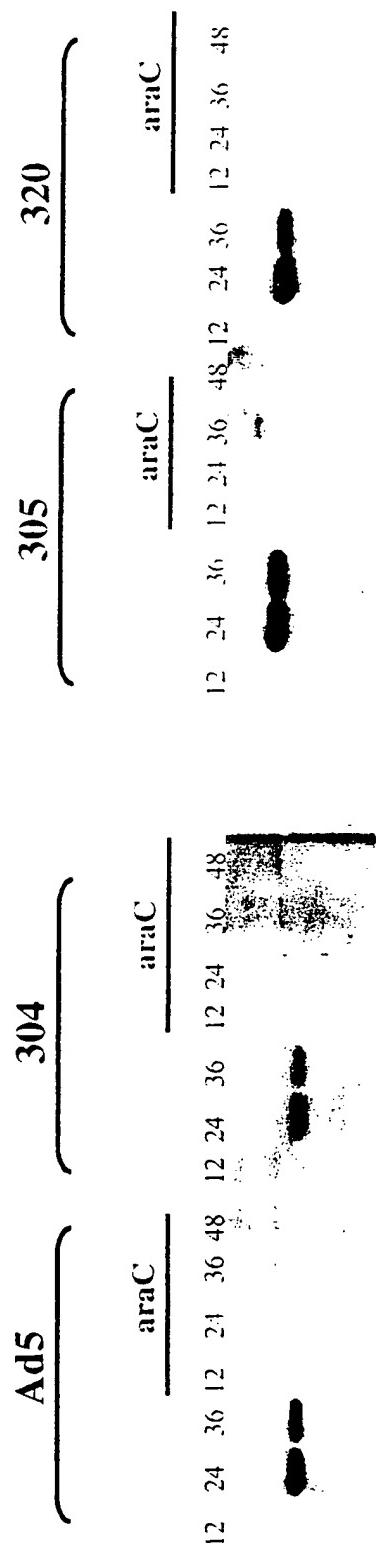


FIGURE 26

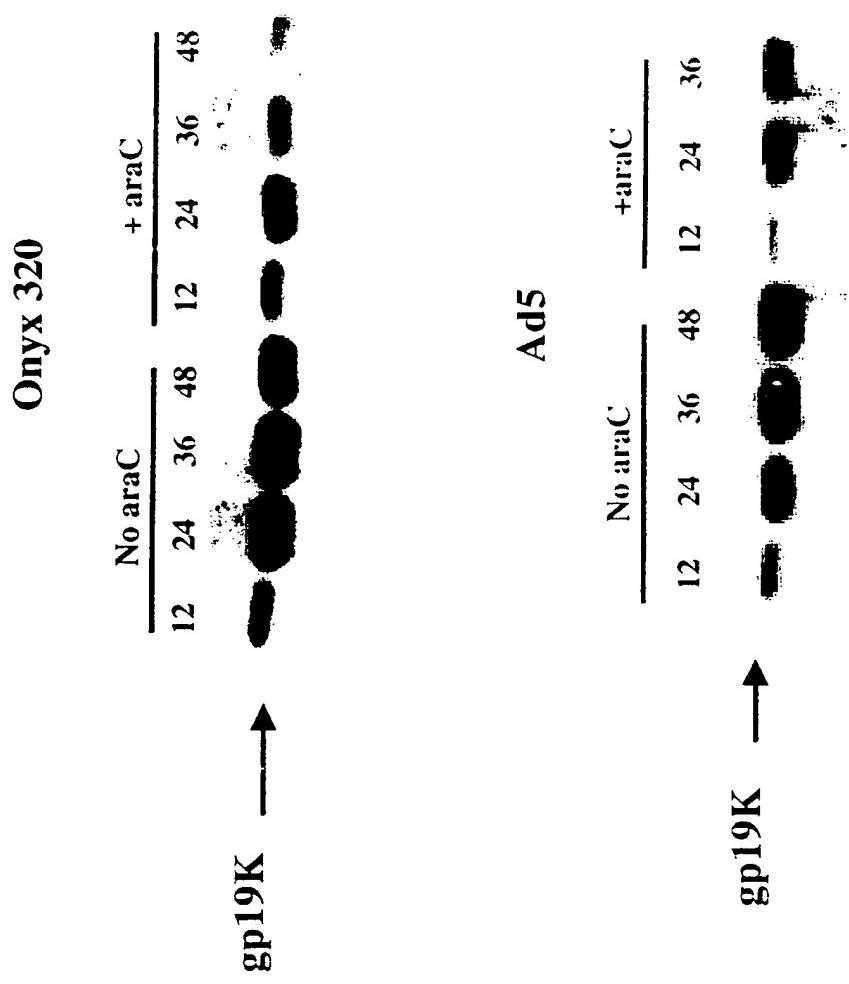
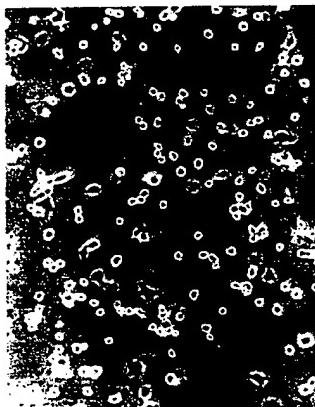
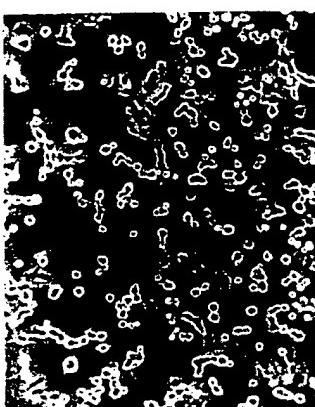
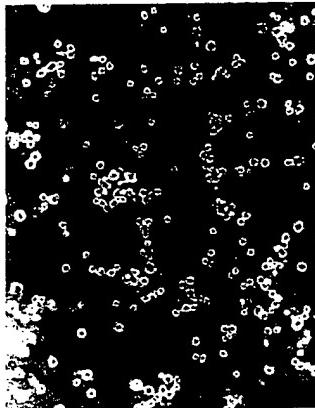
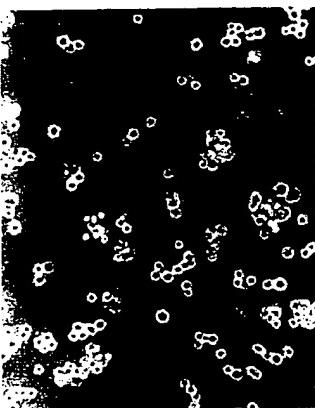


FIGURE 27

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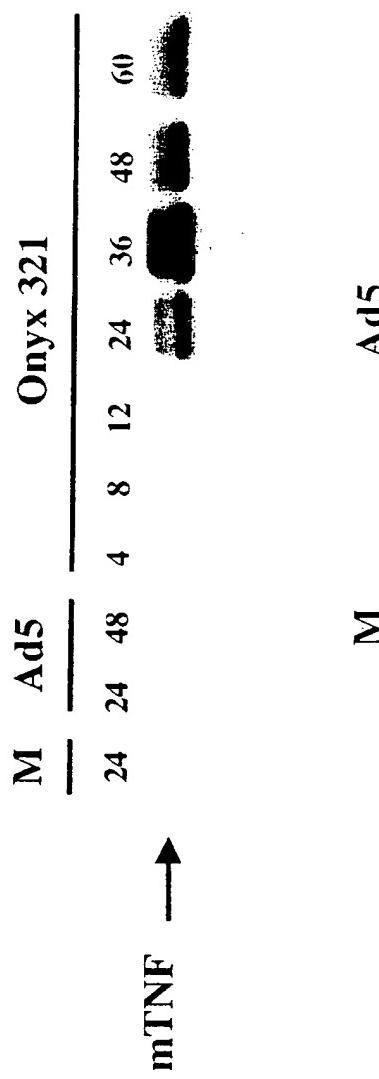
36 hr      48 hr      60 hr



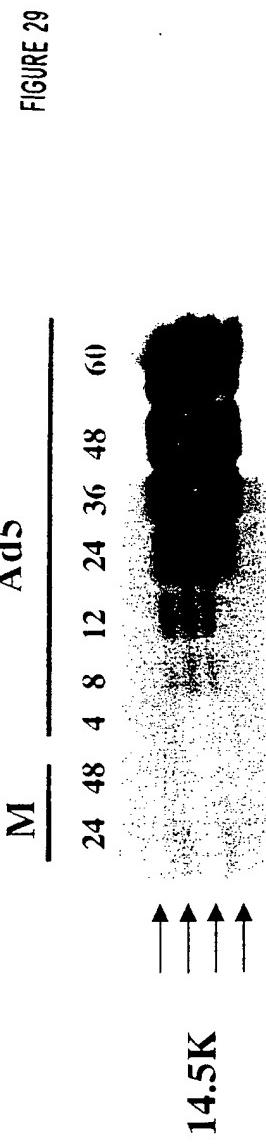
Ad5

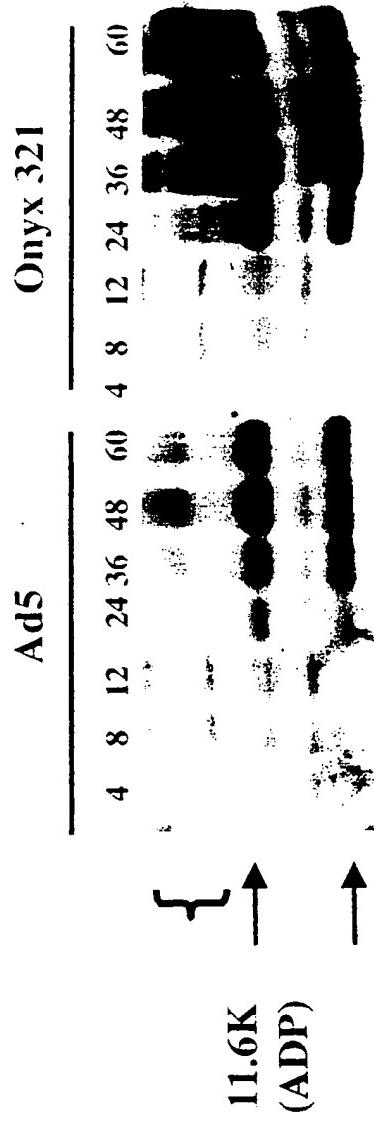
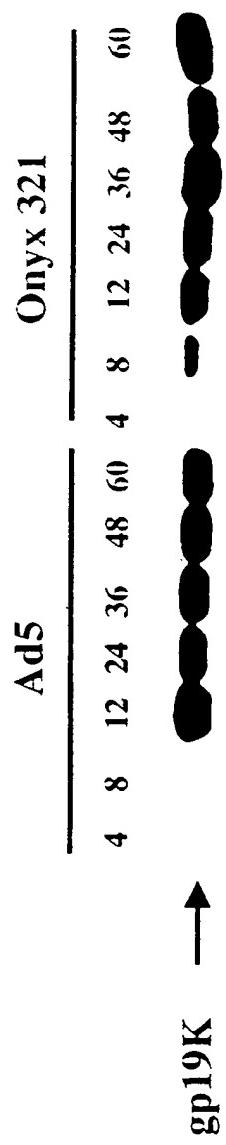
321

FIGURE 28



14.7K    →↑





Synthesis of mTNF in 321-infected cells  
Is Dependent on DNA Replication



FIGURE 31

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Onyx 321 infected-cells  
*minus or plus araC*

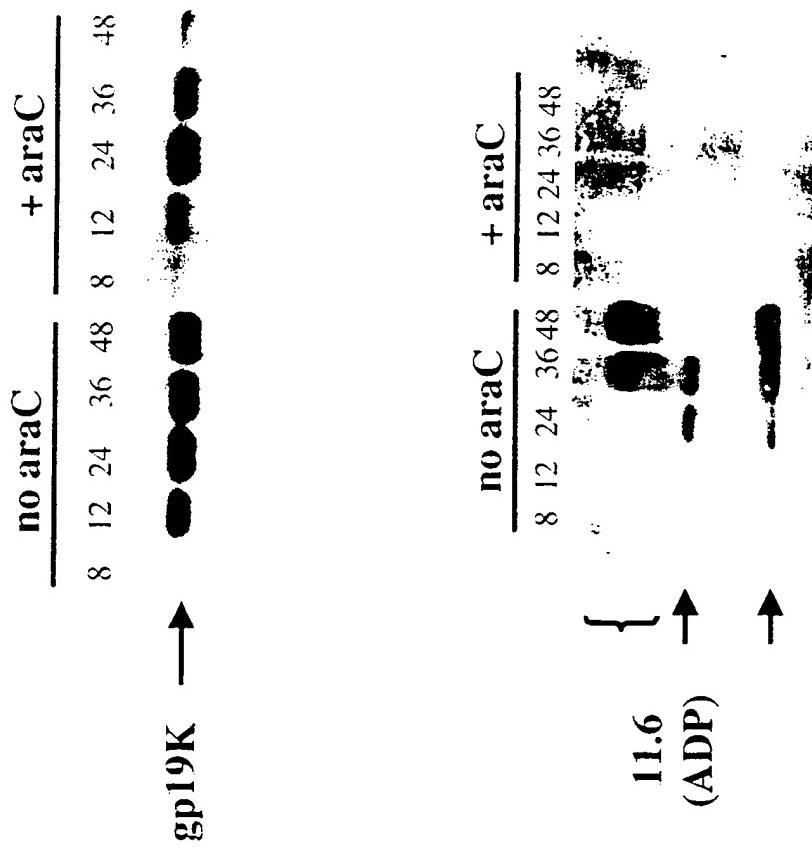


FIGURE 32